REMARKS

Claims 1-14 are pending in this application. Clams 1, 2, 4 and 19 are currently amended.

Claim 1 is amended to track cumulative amendments. Claim 2 is currently amended to delete a pH limitation that was formerly sought to be added to the claims of the issued patent. Claims 4 and 10 are amended to add this pH limitation and delete reference to a concentration range already present in claim 1.

1. Reissue Oath 37 C.F.R §1.175

The Examiner finds that the application was filed without a reissue Oath; however, this is not the case. Although the Oath is captioned "assignee oath, it is signed by the sole inventor John B. Taylor (now deceased). This Oath was filed with the application as and later resubmitted on May 8, 2002 as shown in Tab A attached to this Amendment. The Oath meets the requirements of 37 C.F.R. §1.63 and is merely captioned as an assignee oath due to the assignment of record to Foliar Nutrients Inc.

2. Consent of Assignee

Although there is an objection under 37 C.F.R. §1.172(a) and a requirement to submit the consent, this was filed with the initial application as shown in Tab B attached to this Amendment.

3. Defective Reissue Oath §35 U.S.C. §251

Although claims 1-14 stand rejected for the lack of a proper declaration and consent of the assignee, these are not actually lacking as explained above. Therefore, the rejection appears to be moot.

4. Claim Rejections 35 U.S.C. §112

Claims 4 and 10 stand rejected for not further limiting the base claims from which they depend. This is resolved by the amendment to add a pH range of from 5.0 to 7.0. Support for this limitation is found in the paragraph added to the specification by amendment on June 3, 2008.

Claim 1 stands rejected under 35 USC §112 first paragraph by reason that the second formula as claimed is not always a salt, as the term "salt" is conventionally used. Applicant respectfully traverses the rejection because salt is used in the acceptable manner. Although the basis for rejection is not expressly stated, the Examiner appears to be concerned that one of R1 and R3 must be K. This is not technically correct because

the definition of "salt" permits the use of electropositive radicals, in addition to metals. See Tab C attached to this Amendment.

Claim 1 stands further rejected under 35 U.S.C. §112 first paragraph because the change to alkynyl is not shown. This is resolved in the current version of claim 1.

Claim 2 stands rejected under 37 C.F.R. §112 first paragraph for allegedly having no basis for 20 mM and a pH range. Although the amendment to claim 2 resolves this rejection by deleting the pH range, this range has not been added to claims 4 and 10. Support is as shown in the amendment to the specification filed on June 3, 2008, and this does not constitute new matter, due to the incorporation of this material by reference.

Claims 6-8 and 14-12-14 stand rejected under 35 USC §112 first paragraph because it is said that Item C of column 4 states that solutions of the compounds are mixed and no KOH is evidence. That finding seems untrue. The solutions of Item C include an aqueous solution of H_3PO_3 and KOH (Col. 4 item A) and an aqueous solution of monopotassium phosphate and KOH (Col. 4 Item B.) This is precisely as claimed.

5. Claim Rejections 35 U.S.C. §102

Claim 1 stands rejected as being anticipated by United States Patent No. 4,350,770 issued to Spraker (Spraker '770). Applicant traverses the rejection on bases supported by the Attached Declaration of Phillip W. Spraker (see Tab D) who is the sole inventor named in Spraker '770. Spraker is not actually a reference under 35 U.S.C. §102(b) because when considered in its entirety for the totality of its teachings it does not actually teach the use of KH_2PO_3 on any other phosphonate salt.

The Spraker Declaration attests to the facts that Spraker '770 contains a misprint or error. Specifically, Mr. Spraker never used KH_2PO_3 as taught in column 199 at lines 51-67. IN particular, the KH_2PO_3 there stated should have been identified as KH_2PO_4 (Para. 5) Moreover, this salt formulation was provided to assist bacteria raised on a sole source of carbon (Para. 8), as taught in an article by Curtiss that is cited passim in the text of Spraker '770 (Para 6). This error was readily ascertainable to those of ordinary skill in the art by consultation with Curtiss (Para. 9), as would have been done because one would not normally use a phosphonate salt to encourage the growth of bacteria. Moreover, the use of phosphonate would have mismatched the apparent buffering conjugate to K_2HPO_4.

Thus, Spraker '770 is removed as a reference due to this mistake and consideration of the totality of the reference for what it teaches. It is also removed as a

reference according to other established case law where Applicant has shown that the salt solution identified in Spraker '770 was erroneous and never actually existed. *See In re Kalm*, 378 F.2d 959, 962 (CCPA 1967).

6. Claim Rejections under 35 U.S.C. § 103(a)

Claims 1 and 2 stand rejected under 35 U.S.C. §103(a) as being unpatentable over US 4,350,770 issued to Spraker. The Patent Owner traverses this rejection.

The issue is moot due the elimination of a pH range from claim 2, but see claims 4 and 10. The finding that Spraker shows the basic salt solution as having a pH range of 5.5 to 8.5 is in error because that is the pH of the wastewater stream, not the salt solution disclosed in column 10 at lines 60-68. Spraker '770 means just what it says when it teaches a "basic" salt solution.

As to the allegation that concentration ranges could easily have been optimized, application traverses this finding, which implicates optimization for the purposes taught in Spraker '770, namely, bacterial action to degrease oleaginous wastewater. This would not have been optimization for the purpose of the presently claims composition in the intended environment of use. The optimization analogy fails due to this different and previously unrecognized purpose.

Claims 1, 6 and 12 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Fenn et al, Dolan et al., with evidence exemplified by United States Patent No. 5,070,083 to Barlet. No explanation of this rejection is given, and so Applicant has nothing of substance eon which to base a response, except to say that Fenn and Dolan reach contradictory results and do not achieve the concentrations presently claimed. Moreover, the competitive uptake studies by Griffith, as discussed below, show why the combined references cannot be applied in any manner.

Claims 1, 6 and 12 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Barlet '083 , Ducret et al. 4,139,616, Horriere et al. 5,169,646, Lovett 5,514,200, Vetanovetz et al. 5,390,418 and Smile et al. '89. The Examiner applies Barlet, Ducret and Horrierre to show use of phosphonate salts "as art recognized fungicides," while applying Lovatt and Vetanovitz to show the use of phosphates as "art recognized fertilizers." Smilie at page 924 is said to show the effectiveness of phosphonate salts enhanced with phosphates.

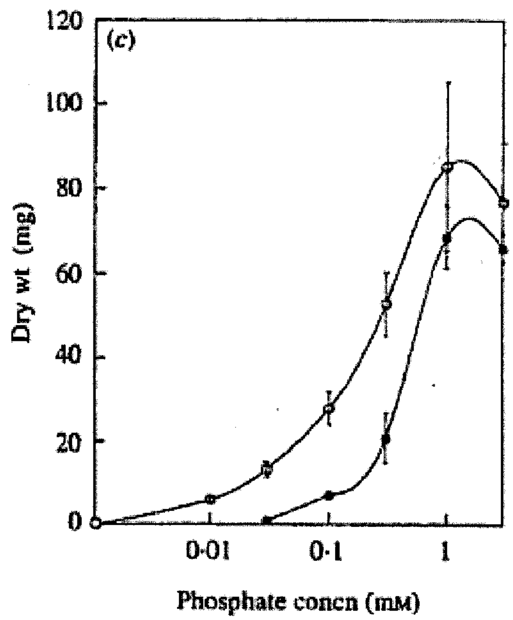
Applicant respectfully traverses this rejection for the reason that it fails to consider the art as a whole for what it teaches, including the teachings of Griffith also implicated in the rejection. Smilie at 924 does not show the effectiveness of phosphonate salts enhanced with phosphates, rather, it shows just the opposite, i.e.:

> Our results also show that the effectiveness of phosphite in providing protection may be influenced by the concentration of phosphate present. The coincident increase in phosphate and <u>breakdown of protection</u> [emphasis added] in tobacco (Figs. 6 and 7) suggests, though does not prove, that the two events are related. If the increased phosphate concentrations reduced the uptake of phosphite into the fungus, breakdown of protection could be expected. We have shown that this is precisely what happens *to P. palmivora* under *in vitro* conditions (J.M. Griffith personal communication). Phosphite entry into *P. palmovora* is directly reduced in the presence of phosphate. There appears to be common transport systems for phosphate and phosphite uptake, and <u>mutually competitive inhibition is exhibited between the two types of anion</u> [emphasis added]. Phosphate has also been shown to inhibit phosphite uptake in *P. citrophthora*, and it was suggested that in this species there might also be two different uptake systems having different affinities for phosphite. The irregular preinfection count of downy mildew by phosphite . . .may be explained by inhibition of phosphite in the fungus in the presence of high phosphate.

Smilie at 924

Thus, Smilie merely confirms the competitive inhibition phenomenon that is also observed in the issued patent in column 2 at lines 57-60. See the mention of a personal note from Griffith in the text above. This does not support a rejection, rather, it is a strong reason favoring the patentability of what is claimed.

In summary form, J. M. Griffith, M.D. Coffey, and B.R. Grant 1993, "Phosphonate inhibition as a function of phosphate concentration in isolates of *Phytophthora palmivora*," J. OF GENERAL MICROBIOL., 139: 2109-2116 shows work on this same *P. palmivora* organism. Fig. 1(c) is replicated below:



○ = Control ● =.treated with 1 mM-phosphonate

The above figure shows that phosphate content above 1 mM inhibits phosphonate uptake. The overall trend as to the diminishing inhibition effect with increasing phosphate content is true with respect to all isolates in the Griffith study, which states on page 2113 that the upper limits for the observed effect were in the range of from 1 mM to 3 mM:

> However, when Pie (phosphate content in the media) did not limit growth, at 1 mM and 3 mM, the P376 and P7228 strains accumulated more Pi (internal phosphate content in the cells) . . . than P113)

Other work by Griffith shows that the metabolic interaction is more complex than one might otherwise imagine. The following Table is copied from J.M Griffith, R. H. Smilie, J.O. Niere and B. R. Grant, 1989, Effect of phosphate on the toxicity of phosphonate *in Phytophthora palmivora*, ARCH. MICROBIOL 152:425-429.

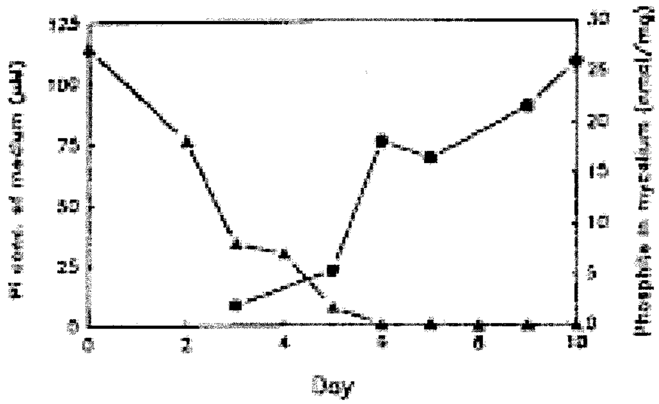


Fig. 1. The uptake of phosphite and the utilization of P_i by *Phytophthora palmivora* during growth in LPR medium containing 1 mM phosphite. P_i and phosphite concentrations were determined by ion chromatography as described in Methods. P_i in medium (▲——▲); phosphite in mycelium (●——●)

Griffith explains the significance of Fig. 1:

> Analysis of the phosphite [phosphonate] content of the mycellium grown in LPR medium in the presence of 1 mM phosphite (the concentration used by Fenn and Coffey in 1984) showed that there was an abrupt increase in the level of phosphite entering the mycellium after Pi [phosphate] had been depleted from the medium at day 6 (Fig. 1).

This is shown above in Fig. 1 where the curve on the left hand side represents diminishing phosphate content in the growth medium, and the curve on the right hand side represents phosphonate that has entered the fungal cells of *P. palmivora*. At these concentrations, the phosphonate does not start to work until the phosphate is depleted. This explains, for example, why "[p]hosphates have also been considered to be a competitive inhibitor for phosphonate assimilation, thus inhibiting the ability of phosphonates to protect against fungus attack." U.S. 5,997,910, col. lines 57-60.

Phosphonates target Phycomycete pathogens, such as Downy Mildew, which appear on the plant leaves. Considering the competitive uptake phenomenon taught by Griffith, it would appear that the phosphonate materials are unavailable to the pathogens until they have first consumed the phosphate materials. This evidence strongly teaches away from the combination that is taught because Griffith shows that the application of phosphonates is useless in combination with phosphates, especially in foliar applications

used to treat the target Phycomycetes. This evidence weighs strongly in favor of nonobviousness because the art shows generally that phosphates should not be mixed with phosphonates to achieve an antifungal effect.

Smilie observes on page 924 that other researchers have reported an opposite effect, i.e.; one where "a different strain of *P. palmivora*, which was extremely sensitive to phosphite *in vitro*, responded in the opposite way to that observed in our study, with phosphate enhancing the effectiveness of phosphite." Smilie then recognizes that the methods used by the other researchers "do not allow direct comparison; with the work described here." Smilie explains this opposite effect as one attributable to a particularly sensitive strain of *P. palmivora*. This only shows that the researchers failed to understand the phenomenon and were unable to produce consistent antifiungal results.

As to the allegation that Applicant has failed to provide any evidence of criticality or unexpected results, that is untrue. Applicant's composition as claimed behaves in an opposite manner as predicted by Griffith and Smilie. Moreover, the working examples show that the claimed concentrations are useful across a broad spectrum of plants and pathogens.

Thus, the great weight of evidence available in the art shows that the competitive uptake phenomenon was known at the time of the present invention. This phenomenon taught away from the claimed use of phosphite in combination with phosphate because phosphate inhibits the uptake of phosphite. Thus, the Phycomycete organism (exemplified by *P. palmivora*) targeted by phosphonate would go largely unaffected due to phosphate inhibiting the uptake of phosphonate. This would be akin to mixing poison with a poison antidote. The expectation was that a majority of phosphite applied in combination with phosphate would likely be wasted because the pathogens at issue in the intended environment of use selectively uptake phosphate to the exclusion of phosphite whenever phosphate is present. The prior art strongly suggests that the application of phosphonate in combination with phosphate would not hit the intended target.

Claims 1,3-5, and 9-11 stand rejected under 35 USC §103(a) as being unpatentable over Thyzy et al. 4,075,324 in view of Reuveni et al J. Phyto 141, Reuveni et al. J. Phyto 44, Dunstan et al, Fenn Dissertation, Walker, and the Fluid Fertilizer Manual. The only references combining PO_3 and PO_4 salts are Walker and Fenn, which use concentrations of 10 mM or less. Griffith, as discussed above, shows that these concentrations suffer from inhibition due to competitive uptake, and so they presumptively

lack efficacy. The other references cited only use PO_3 and PO_4 salts in isolation not in combination. Thus, one would not have known that the claimed solution cold be effective in its application, since the art as illustrated by Smilie and Griffith teaches away from the combination for the reasons explained above.

While some of the references may disclose use of PO_3 salts as fertilizers, the uptake of PO_3 also would be competitively inhibited by the addition of PO_4 and therefore contraindicated. Use of the more general Fluid Fertilizer Manual to allegedly show motivation to combine fungicides or fertilizers is meaningless and completely overcome by the more specific contraindication as per Griffith and Smilie.

For the foregoing reasons, Applicant's attorney respectfully submits that the claims are worthy of allowance. Applicant believes no additional fees other than the 3-month extension of time are due, however, if any additional fee is deemed necessary in connection with this Response, please charge Deposit Account No. 12–0600.

Respectfully submitted,

Dan Cleveland, Reg. No. 36,106
Lathrop & Gage LLP
4845 Pearl East Circle, Suite 201
Boulder, CO 80301
(720) 931-3012 (phone)
(720) 931-3001 (fax)

TAB A

REISSUE APPLICATION DECLARATION BY THE ASSIGNEE	Docket Number (optional) 396542

I hereby declare that:

My residence and post office address and citizenship are stated below next to my name.

I am authorized to act on behalf of the following assignee: Foliar Nutrients, Inc.

and the title of my position with said assignee is: President

The entire title to the patent identified below is vested in said assignee.

Name of Patentee(s):
John B. Taylor

Patent Number	Date of Patent Issued
5,997,910	December 7, 1999

Title of Invention
PLANT FERTILIZER COMPOSITIONS CONTAINING PHOSPHONATE AND PHOSPHATE SALTS AND DERIVATIVES THEREOF

I believe said patentee(s) to be the original, first and sole/joint inventor(s) of the subject matter which is

Described and claimed in said patent, for which a reissue patent is sought on the invention entitled PLANT FERTILIZER COMPOSITIONS CONTAINING PHOSPHONATE AND PHOSPHATE SALTS AND DERIVATIES THEREOF.

the specification of which

☒ is attached hereto.
☐ was filed on as reissue application number /
and was amended on
(If applicable)

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I verily believe the original patent to be wholly or partly inoperative or invalid, for the reasons described below. (Check all boxes that apply.)

☒ by reason of a defective specification or drawing.

☒ by reason of the patentee claiming more or less than he had the right to claim in the patent.

☐ by reason of other errors.

At least one error upon which reissue is based is described as follows: that the "millimole" language at column 3, line 52 and column 4, line 51 is an obvious typographical error in that "millimole" was intended to be "millimolar" in the specified concentration range as "millimolar" is a unit of concentration while "millimole" is not;

that the phosphonate salt formula at column 8, lines 27-35 contains an obvious typographical error in that the bond between P and R2 has to be a single bond rather than a double bond as shown;

that the specification at column 8, lines 49-50 and claim 1 at column 10, line 26 contains an obvious typographical error in that the term "alkinyl" was clearly intended to be "alkynyl"; and

that the Patentee did not claim all that he had the right to claim in the patent in that the method of the invention was not claimed in an alternative fashion reciting the materials used in the preparation of the first and second salts, such alternative method being clearly supported in the specification of the patent.

Patentee respectfully submits that the statements of errors herein comply with the requirement of 37 C.F.R. § 1.175 (a)(1) of identifying the errors in the original patent relied upon as the basis of reissue. However, Patentee respectfully submits that the statements above do not constitute an admission that those errors render the original patent claims inoperative or invalid.

[Attach additional sheets, if needed.]

All errors corrected in this reissue application arose without any deceptive intention on the part of the applicant.

EXHIBIT A

(REISSUE APPLICATION DECLARATION BY THE ASSIGNEE)	Docket Number (Optional) 366542

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Name(s)	Registration Number
Kenneth D. Goetz	32,696
William A. Rudy	34,915
Peter C. Knops	37,659
Janelle D. Strode	34,738

Correspondence Address: Direct all communications about the application to:

☐ Customer Number [ ] → Place Customer Number Bar Code Label Here

Type Customer Number Here

OR

☒ Firm or Individual Name	LATHROP & GAGE LC		
Address	2345 Grand Boulevard		
Address	Suite 2800		
City	Kansas City	State	Missouri	Zip	64108
Country	United States of America		
Telephone	(816) 460-5849	Fax	(816) 292-2001

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this declaration is directed.

Full name of person signing (given name, family name)
John B. Taylor

Signature: John B. Taylor | Date: 10/19/01

Address of Assignee
320 First Avenue, N.E., Cairo, Georgia 31728

Patentee John B. Taylor	Citizenship United States of America

Residence/Post Office Address
1420 Lemon Street, DeLand, Florida ~~32730~~ 32720

Patentee	Citizenship

Residence/Post Office Address

☐ Additional Patentees are named on separately numbered sheets attached hereto.

TAB B

PTO/SB/96 (08-00)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: John B. Taylor

Application No./Patent No.: 5,997,910 Filed/Issue Date: December 7, 1999

Entitled: Plant Fertilizer Compositions Containing Phosphonate and Phosphate Salts And Derivatives Thereof

Foliar Nutrients, Inc , a Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. ☒ the assignee of the entire right, title, and interest; or
2. ☐ an assignee of an undivided part interest

in the patent application/patent identified above by virtue of either:

A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the Patent and Trademark Office at Reel 010756, Frame 0022, or for which a copy thereof is attached.

OR

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as shown below:

1. From: ____ To: ____
The document was recorded in the United States Patent and Trademark Office at Reel ____, Frame ____, or for which a copy thereof is attached.

2. From: ____ To: ____
The document was recorded in the United States Patent and Trademark Office at Reel ____, Frame ____, or for which a copy thereof is attached.

3. From: ____ To: ____
The document was recorded in the United States Patent and Trademark Office at Reel ____, Frame ____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet.

☐ Copies of assignments or other documents in the chain of title are attached.
[NOTE: A separate copy (i.e., the original assignment document or a true copy of the original document) must be submitted to Assignment Division in accordance with 37 CFR Part 3, if the assignment is to be recorded in the records of the USPTO. See MPEP 302.8]

The undersigned (whose title is supplied below) is empowered to sign this statement on behalf of the assignee.

10/19/01
Date

Monty C. Ferrell
Signature

Monty C. Ferrell
Typed or printed name

President
Title

TAB C

SALT

Also found in: Medical, Acronyms, Idioms, Encyclopedia, Wikipedia, Hutchinson

0.04 sec.

SALT (sôlt)
abbr.
Strategic Arms Limitation Talks

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salt (sôlt)
n.
1. A colorless or white crystalline solid, chiefly sodium chloride, used extensively in ground or granulated form as a food seasoning and preservative. Also called *common salt*, *table salt*.
2. A chemical compound formed by replacing all or part of the hydrogen ions of an acid with metal ions or electropositive radicals.
3. **salts** Any of various mineral salts used as laxatives or cathartics.
4. **salts** Smelling salts.
5. Epsom salts. Often used in the plural.
6. An element that gives flavor or zest.
7. Sharp lively wit.
8. *Informal* A sailor, especially when old or experienced.
9. A saltcellar.

adj.
1. Containing or filled with salt: *a salt spray; salt tears.*
2. Having a salty taste or smell: *breathed the salt air.*
3. Preserved in salt or a salt solution: *salt mackerel.*
4.
   a. Flooded with seawater.
   b. Found in or near such a flooded area: *salt grasses.*

tr.v. **salt·ed, salt·ing, salts**
1. To add, treat, season, or sprinkle with salt.
2. To cure or preserve by treating with salt or a salt solution.
3. To provide salt for (deer or cattle).
4. To add zest or liveliness to: *salt a lecture with anecdotes.*
5. To give an appearance of value to by fraudulent means, especially to place valuable minerals in (a mine) for the purpose of deceiving.

Phrasal Verbs:
salt away
To put aside; save.
salt out
To separate (a dissolved substance) by adding salt to the solution.

Idioms:
salt of the earth
A person or group considered as the best or noblest part of society.
worth (one's) salt
Efficient and capable.

[Middle English, from Old English `sealt`; see `sal-` in Indo-European roots.]

---

salt [sɔːlt]
n
1. (Chemistry / Elements & Compounds) a white powder or colourless crystalline solid,

consisting mainly of sodium chloride and used for seasoning and preserving food
2. (Chemistry / Elements & Compounds) *(modifier)* preserved in, flooded with, containing, or growing in salt or salty water *salt pork salt marshes*
3. (Chemistry) *Chem* any of a class of usually crystalline solid compounds that are formed from, or can be regarded as formed from, an acid and a base by replacement of one or more hydrogen atoms in the acid molecules by positive ions from the base
4. liveliness or pungency *his wit added salt to the discussion*
5. dry or laconic wit
6. (Transport / Nautical Terms) a sailor, esp one who is old and experienced
7. (Cookery) short for saltcellar
rub salt into someone's wounds to make someone's pain, shame, etc., even worse
salt of the earth a person or group of people regarded as the finest of their kind
with a grain (*or* pinch) of salt with reservations; sceptically
worth one's salt efficient; worthy of one's pay

vb (tr)
1. (Cookery) to season or preserve with salt
2. to scatter salt over (an icy road, path, etc.) to melt the ice
3. to add zest to
4. (Cookery) *(often foll by down or away)* to preserve or cure with salt or saline solution
5. (Chemistry) *Chem* to treat with common salt or other chemical salt
6. (Life Sciences & Allied Applications / Agriculture) to provide (cattle, etc.) with salt
7. (Mining & Quarrying) to give a false appearance of value to, esp to introduce valuable ore fraudulently into (a mine, sample, etc.)

adj
1. (Life Sciences & Allied Applications / Physiology) not sour, sweet, or bitter; salty
2. *Obsolete* rank or lascivious (esp in the phrase **a salt wit**) See also salt away, salt out, salts

[Old English *sealt;* related to Old Norse, Gothic *salt,* German *Salz,* Lettish *sāls,* Latin *sāl,* Greek *hals*]

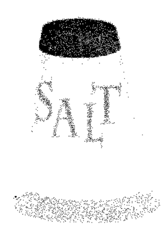
saltish *adj*
saltless *adj*
saltlike *adj*
saltness *n*

SALT [sɔ : lt]
n acronym for
(Government, Politics & Diplomacy) Strategic Arms Limitation Talks *or* Treaty

salt (sôlt)
1. Any of a large class of chemical compounds formed when a positively charged ion (a cation) bonds with a negatively charged ion (an anion), as when a halogen bonds with a metal. Salts are water soluble; when dissolved, the ions are freed from each other, and the electrical conductivity of the water is increased. See more at complex saltdouble saltsimple salt
2. A colorless or white crystalline salt in which a sodium atom (the cation) is bonded to a chlorine atom (the anion). This salt is found naturally in all animal fluids, in seawater, and in underground deposits (when it is often called *halite*). It is used widely as a food seasoning and preservative. Also called *common salt, sodium chloride, table salt. Chemical formula:* **NaCl.**

Thesaurus

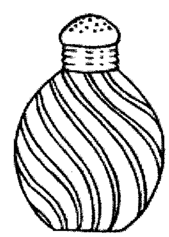
Legend: Synonyms Related Words Antonyms



Noun 1. **salt** - a compound formed by replacing hydrogen in an acid by a metal (or a radical that acts like a metal)
acetate, ethanoate - a salt or ester of acetic acid
citrate - a salt or ester of citric acid
arsenate - a salt or ester of arsenic acid
chlorate - any salt of chloric acid
dibasic salt - a salt derived by replacing two hydrogen atoms per molecule
hypochlorite - any salt or ester of hypochlorous acid
pyrophosphate - a salt or ester of pyrophosphoric acid
sulfonate - a salt of sulphonic acid
lactate - a salt or ester of lactic acid
perchlorate - a salt of perchloric acid
alkali - a mixture of soluble salts found in arid soils and some bodies of water;

detrimental to agriculture
ammonium chloride, sal ammoniac - a white salt used in dry cells
benzoate - any salt or ester of benzoic acid
borate - a salt or ester of boric acid
borosilicate - a salt of boric and silicic acids
calcium lactate - a white crystalline salt made by the action of lactic acid on calcium carbonate; used in foods (as a baking powder) and given medically as a source of calcium
calcium octadecanoate, calcium stearate - an insoluble calcium salt of stearic acid and palmitic acid; it is formed when soap is mixed with water that contains calcium ions and is the scum produced in regions of hard water
carbamate - a salt (or ester) of carbamic acid
carbonate - a salt or ester of carbonic acid (containing the anion CO3)
fulminate - a salt or ester of fulminic acid
chromate - any salt or ester of chromic acid
chemical compound, compound - (chemistry) a substance formed by chemical union of two or more elements or ingredients in definite proportion by weight
cyanide - an extremely poisonous salt of hydrocyanic acid
potassium bromide - a white crystalline salt (KBr) used as a sedative and in photography
potassium chlorate - a white salt (KClO3) used in matches, fireworks, and explosives; also used as a disinfectant and bleaching agent
potassium dichromate - an orange-red salt used in making dyes and in photography
ferricyanide - salt of ferricyanic acid obtained by oxidation of a ferrocyanide
ferrocyanide - salt of ferrocyanic acid usually obtained by a reaction of a cyanide with iron sulphate
fluoroboride - a salt of fluoroboric acid
fluosilicate - salt of fluosilicic acid
glutamate - a salt or ester of glutamic acid
halide - a salt of any halogen acid
isocyanate - a salt or ester of isocyanic acid
calcium chloride - a deliquescent salt; used in de-icing and as a drying agent
calcium sulfate, calcium sulphate - a white salt (CaSO4)
manganate - a salt of manganic acid containing manganese as its anion
chrome alum - a violet-colored salt used in hide tanning and as a mordant in dyeing
tartrate - a salt or ester of tartaric acid
oxalacetate, oxaloacetate - a salt or ester of oxalacetic acid
oxalate - a salt or ester of oxalic acid
permanganate - a dark purple salt of permanganic acid; in water solution it is used as a disinfectant and antiseptic
inorganic phosphate, orthophosphate, phosphate - a salt of phosphoric acid
polyphosphate - a salt or ester of polyphosphoric acid
acrylate, propenoate - a salt or ester of propenoic acid
salicylate - a salt of salicylic acid (included in several commonly used drugs)
double salt - a solution of two simple salts that forms a single substance on crystallization
bile salt - a salt of bile acid and a base; functions as an emulsifier of lipids and fatty acids
Glauber's salt, Glauber's salts - (Na2SO4.10H2O) a colorless salt used as a cathartic
cream of tartar, potassium bitartrate, potassium hydrogen tartrate, tartar - a salt used especially in baking powder

2. **salt** - white crystalline form of especially sodium chloride used to season and preserve food
table salt, common salt
flavorer, flavoring, flavourer, flavouring, seasoning, seasoner - something added to food primarily for the savor it imparts



3. **SALT** - negotiations between the United States and the Union of Soviet Socialist Republics opened in 1969 in Helsinki designed to limit both countries' stock of nuclear weapons
Strategic Arms Limitation Talks
4. **salt** - the taste experience when common salt is taken into the mouth
salinity, saltiness
gustatory perception, gustatory sensation, taste, taste perception, taste sensation - the sensation that results when taste buds in the tongue and throat convey information about the chemical composition of a soluble stimulus; "the candy left him

with a bad taste"; "the melon had a delicious taste"

Verb 1. **salt** - add salt to

cookery, cooking, preparation - the act of preparing something (as food) by the application of heat; "cooking can be a great art"; "people are needed who have experience in cookery"; "he left the preparation of meals to his wife"

flavor, flavour, season - lend flavor to; "Season the chicken breast after roasting it"

2. **salt** - sprinkle as if with salt; "the rebels had salted the fields with mines and traps"

splash, sprinkle, splosh - cause (a liquid) to spatter about, especially with force; "She splashed the water around her"

3. **salt** - add zest or liveliness to; "She salts her lectures with jokes"

spice, spice up - make more interesting or flavorful; "Spice up the evening by inviting a belly dancer"

4. **salt** - preserve with salt; "people used to salt meats on ships"

cookery, cooking, preparation - the act of preparing something (as food) by the application of heat; "cooking can be a great art"; "people are needed who have experience in cookery"; "he left the preparation of meals to his wife"

preserve, keep - prevent (food) from rotting; "preserved meats"; "keep potatoes fresh"

Adj. 1. **salt** - (of speech) painful or bitter; "salt scorn"- Shakespeare; "a salt apology"

sharp - keenly and painfully felt; as if caused by a sharp edge or point; "a sharp pain"; "sharp winds"

salt

noun

1. seasoning, sodium chloride, table salt, rock salt *a pinch of salt*
2. sailor, marine, seaman, mariner, tar *(informal)*, hearty *(informal)*, navigator, sea dog, seafarer, matelot *(slang, chiefly Brit.)*, Jack Tar, seafaring man, lascar, leatherneck *(slang)* *`Did he look like an old sea salt?' I asked, laughing.*

verb

add salt to, flavour with salt *Salt the stock to your taste.*

adjective

salty, salted, saline, brackish, briny *Put a pan of salt water on to boil.*

rub salt into the wound make something worse, add insult to injury, fan the flames, aggravate matters, magnify a problem *I had no intention of rubbing salt into his wounds.*

with a grain *or* pinch of salt sceptically, suspiciously, cynically, doubtfully, with reservations, disbelievingly, mistrustfully *You have to take these findings with a pinch of salt.*

Translations

Select a language: ----------------------

salt

n **salt** [so : lt]

1 (alsocommon salt) sodium chloride, a white substance frequently used for seasoning *The soup needs more salt.*

2 any other substance formed, like common salt, from a metal and an acid.

3 a sailor, especially an experienced one *an old salt.*

adj

containing, tasting of, preserved in salt *salt water; salt pork.*

v

to put salt on or in *Have you salted the potatoes?*

adj **salted**

(negativeunsalted) containing or preserved with salt *salted butter; salted beef.*

n **saltness**

adj **salty**

containing or tasting of salt *Tears are salty water.*

n **saltiness**

bath salts

a usually perfumed mixture of certain salts added to bath water.

the salt of the earth

a very good or worthy person *People like her are the salt of the earth.*

take (something) with a grain/pinch of salt

to receive (a statement, news etc) with a slight feeling of disbelief *I took his story with a pinch of salt.*

SALT →

Mentioned in

common salt
complex salt
salt out
salted
salting
table salt

References in classic literature

Schemes of Captain Bonneville The Great **Salt** Lake Expedition to explore it Preparations for a journey to the Bighorn
The Adventures of Captain Bonneville, U.S.A., in the Rocky Mountains and the far West by *Irving, Washington* **View in context**

His place of abode was in Staffordshire, on a morsel of freehold land of his own--appropriately called **Salt** Patch.
Man And Wife by *Collins, Wilkie* **View in context**

To this place caravans of Abyssinia are continually resorting, to carry **salt** into all parts of the empire, which they set a great value upon, and which in their country is of the same use as money.
A Voyage to Abyssinia by *Lobo, Father* **View in context**

More results

Dictionary/thesaurus browser

Salso-acid
Salsoda
Salsola
Salsola kali
Salsola kali tenuifolia
Salsola soda
salsuginous
SALT
Salt acid
salt away
salt bath
Salt block
Salt bottom
salt cake
salt cod

Full browser

Salsoda
Salsola
Salsola
Salsola
Salsola Arbuscula
Salsola kali
Salsola kali
Salsola kali
Salsola kali tenuifolia
Salsola kali tenuifolia
Salsola komarovi
Salsola komarovi
Salsola komarovii
Salsola komarovii
Salsola soda
Salsola soda
Salsola soda
Salsola tuberculatiformis, Salsola tuberculata var. tomentosa
Salsola tuberculatiformis, Salsola tuberculata var. tomentosa
Salsola tuberculatiformis, Salsola tuberculata var. tomentosa
salsoline
SALSRA
salsuginous
SALSUS
SALT
salt & pepper appearance
Salt & Pepper to Taste
Salt (compound)
Salt (compound)
Salt (compound)
Salt (compound)
Salt (food)
Salt (food)
Salt (food)
Salt (food)

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Word / Article Starts with Ends with Text

TAB D

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant:	John B. Taylor	Examiner:	Levy, Neil S
Reissue Application No.:	10/040,046	Group Art Unit:	1615
Filed:	October 19, 2001	Confirmation No..:	1834
For:	PLANT FERTILIZER COMPOSITIONS CONTAINING PHOSPHONATE AND PHOSPHATE SALTS AND DERIVATIVES THEREOF		

October 28, 2010

DECLARATION OF PHILIP W. SPRAKER PRESENTED UNDER 37 C.F.R. §1.132

1. My name is Philip W. Spraker, and I am the only inventor named in United States Patent No. 4,350,770 (now expired). A true copy of this patent is attached as Exhibit A to this Declaration.

2. I currently reside at 5354 East Lee Highway, Max Meadows, Virginia 24360.

3. I assigned the patent of Exhibit A to Sybron Corporation of Rochester, New York. I left the employment of Sybron in 1983. I have retained no records of the studies leading to this patent and have never gained monetarily from the claims granted in this patent.

4. I have read and understood the office action dated May 3, 2010, which is attached as Exhibit B to this Declaration. I understand that Pages 5 and 6 of Exhibit B state rejections of the pending claims in context of Exhibit A, including rejections under 35 U.S.C. §102 (anticipation) and 35 U.S.C. §103 (obviousness). This Declaration is provided to present the Examiner with additional facts for consideration upon review of these rejections.

5. I have formed an opinion that the salt solution described in Example 1 of Exhibit A at column 10 lines 51-67 contains a misprint that is identifiable as such in context of the remainder of the document. Specifically, the identification of KH_2PO_3 at line 63 should be printed as --KH_2PO_4—to reflect what I actually used and did.

6. Exhibit C attached to this Declaration is a true copy of Curtiss, III, J. Bact. 89 28-40 (1965). This is the reference identified as providing the content of "minimal salts" for the support of such microorganisms as *Pseudomonas aeruginosa* HCP in these portions of Exhibit A: column 3 at lines 59-60; column 6 at line 36; column 6 at lines 53-54; column 7 at lines 18-19, and column 12 at line 9.

7. My review of Exhibit C has determined that the salt solution described on page 28 thereof is identical to the salt solution described in Exhibit A at column 10, lines 51-67, except for the misprint previously described. Specifically, the identification of KH_2PO_3 at line 63 should have been printed as --KH_2PO_4 -- in the issued patent.

8. The error mentioned above is easily ascertainable as such. Example 1 of Exhibit A is of bacterial growth on a "sole source of carbon." The numerical salt solution concentration percentages are exactly the same as referenced in Exhibit C, which as shown above contains KH_2PO_4, not KH_2PO_3. This salt mixture is used because it is well known that bacterial growth mediums evaluating 'sole source of carbon' metabolism must contain orthophosphates (PO_4) that support bacterial metabolism. Therefore, KH_2PO_3 would not have been used, and this is clearly ascertainable by practitioners in this field. Also, the error is further evident because KH_2PO_3 would not have been used along with K_2HPO_4 for buffering the medium, as the proper buffering conjugate to K_2HPO_4 would be KH_2PO_4.

9. For the reasons outlined above, Exhibit A is known to contain a misprint that is readily ascertainable as such by practitioners who would then consult Exhibit C for the correct salt mixture confirming the use of KH_2PO_4, not KH_2PO_3. Thus, it is my opinion and belief that, to the eye of persons of ordinary skill in the art, Exhibit A when considered for the totality of its teaching as a whole describes only the use of KH_2PO_4, not KH_2PO_3.

10. Exhibit D attached to this Declaration is a copy of my Curriculum Vitae outlining my education and employment history.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false

statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date: 11/1/2010 By: Philip W. Spraker

Philip W. Spraker

Philip Welsh Spraker
5354 East Lee Highway
Max Meadows, VA 24360

Date of Birth: May 15, 1943

EDUCATION:

Graduated Rural Retreat High School, 1961.
Associate in Science, Wytheville Community College 1973
BS in Biochemistry, Virginia Polytechnic Institute & State University, 1975.

EMPLOYMENT HISTORY:

US Army March 1962 - September 1965
Construction: Heavy Equipment Operator, 1966 – March 1970
Injury from Automobile Accident & Convalescence, March 1970 – March 1971
Student, March 1971 – June 1975

Sybron Corporation, Biochemical Division, Salem, VA, 1975 – 1983
- R&D Technician – Development of Bacterial Cultures for the Breakdown of Industrial Chemicals
- Field Service Technician
- Operations/Facility Manager

Microbe Masters, Inc (Texas Based), September 1983 – April 1985
- Technical Services – Biological Waste Treatment

Self Employed (Texas Based Consultant), April 1985 – October 1985
- Consultant – Biological Waste Treatment

Analytichem International, Texas Based, October 1985 – October 1986
- Sales Representative for Sample Preparation Materials before chromatographic analysis

Self Employed, Texas Based, October 1986– October 1987
- Consultant in field of Microbiology
- Commissioned sales representative for Worldwide Monitoring Inc., for Sample Preparation Materials

UCT, LLC., Basic Manufacturer of Sample Preparation Materials, October 1987 – To present
- UCT was previously named Worldwide Monitoring Inc. & United Chemical Technologies, Inc.,
- Texas Sales Representative, 1987 - 1990
- Vice President of Operations at Bristol, PA 1990 – 1993
- Vice President of Sales and Marketing at Bristol, PA 1993 – 1996
- Sales Representative (Virginia Based) – Mid West and Southeast Territories 1996 – 2001
- Operations Manager (At Lewistown & Bristol, PA Locations), 2001 – 2002
- Sales Manager (Virginia Based), 2002 – 2004
- Key Account Manager (Virginia Based), 2004 - Present

FAX COVER SHEET

TO: Robert Adair
Executive Director
Florida Research Center for Agricultural Sustainability, Inc.
7055 33rd Street
Vero Beach, Florida 32966

FROM: Philip Welsh Spraker
5354 East Lee Highway
Max Meadows, VA 24360

DATE: November 1, 2010

PAGES: 4

Attached is: DECLARATION OF PHILIP W. SPRAKER, PRESENTED UNDER 37 C.F.R. §1.132. Thank you for your offer of compensation for time and expenses. At this point, none is necessary. However, by accepting and using this document, you and your organization accept responsibility for my time, expenses and costs that might occur from future actions relating to this document.

Philip W. Spraker

United States Patent [19]

Spraker

[11] **4,350,770**

[45] *** Sep. 21, 1982**

[54] **MICROBIOLOGICAL PROCESS FOR REMOVING OLEAGINOUS MATERIAL FROM WASTEWATER AND MICROBIOLOGICAL COMBINATION CAPABLE OF SAME**

[75] Inventor: **Philip W. Spraker,** Troutville, Va.

[73] Assignee: **Sybron Corporation,** Rochester, N.Y.

[*] Notice: The portion of the term of this patent subsequent to Sep. 8, 1998, has been disclaimed.

[21] Appl. No.: **261,394**

[22] Filed: **May 7, 1981**

Related U.S. Application Data

[63] Continuation of Ser. No. 4,241, Jan. 17, 1979, Pat. No. 4,288,545.

[51] **Int. Cl.**³ **C12N 1/20**

[52] **U.S. Cl.** **435/253;** 435/172; 435/833; 435/834; 435/835; 435/836; 435/837; 435/839; 435/875; 435/877

[58] **Field of Search** .... 435/833, 834, 42, , 435/839, 19, 172, 271, 272, 804, 835–837, 875, 877; 210/606, 611

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,843,517	10/1974	McKinney et al.	210/611
3,871,957	3/1975	Mohan et al.	210/611
4,146,470	3/1979	Mohan et al.	210/611
4,159,944	7/1979	Erickson et al.	210/611

Primary Examiner—R. B. Penland

Attorney, Agent, or Firm—Sughrue, Mion, Zinn, Macpeak & Seas

[57] **ABSTRACT**

A process for removing oleaginous materials containing those of animal origin from wastewater comprising treating wastewater containing oleaginous material with a microbial combination of:

(a) a microorganism of the strain *Pseudomonas aeruginosa* mutant $SGRR_2$; and

(b) at least one of:

(i) a microorganism of the genus Bacillus; and

(ii) a microorganism of the genus Pseudomonas other than the strain *Pseudomonas aeruginosa* mutant $SGRR_2$;

and the microbial combination of:

(a) a microorganism of the strain *Pseudomonas aeruginosa* mutant $SGRR_2$; and

(b) at least one of:

(i) a microorganism of the genus Bacillus; and

(ii) a microorganism of the genus Pseudomonas other than the strain *Pseudomonas aeruginosa* mutant $SGRR_2$.

6 Claims, No Drawings

EXHIBIT

tabbles®

A

MICROBIOLOGICAL PROCESS FOR REMOVING OLEAGINOUS MATERIAL FROM WASTEWATER AND MICROBIOLOGICAL COMBINATION CAPABLE OF SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of co-pending application Ser. No. 4,241, filed Jan. 17, 1979, now U.S. Pat. No. 4,288,545.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a process of removing oleaginous material from domestic, municipal and industrial wastewater and, more specifically, to a method for removing oleaginous material containing oleaginous material of animal origin from wastewater containing the same using a microbial combination of a novel mutant of *Pseudomonas aeruginosa* and at least one other microorganism of the genus Bacillus or of the genus Pseudomonas other than the novel mutant *Pseudomonas aeruginosa* whereby oleaginous material in the wastewater is degraded and thereby removed from the wastewater. Further, this invention relates to a novel microbial combination of the novel mutant of *Pseudomonas aeruginosa* in combination with at least one other microorganism of the genus Bacillus or of the genus Pseudomonas other than the novel mutant of *Pseudomonas aeruginosa*.

2. Description of the Prior Art

Fats and greases of either animal or vegetable origin, particularly of animal origin, have historically caused problems in systems for handling wastewater. Breakdown of these fats and greases is very slow, and due to their inherent nature of being water insoluble, fats and greases have a tendency to coat or completely clog drain or treatment systems. Disposal of fats is also hindered. Because they have a low specific gravity and a high melting point, fats float and solidify, causing difficulties in closed treatment systems such as in domestic septic tanks and in buried pipes where the grease and other waste become trapped and build up.

Further, problems arise in municipal and industrial systems due to the presence of grease and fats and, even in aerated waste handling lagoons and other systems as used by meat and poultry processors, the natural degradation process is often too slow, resulting in thick layers of grease and fat which build up to critical levels or carry through the system undigested.

As a result of the difficulty arising due to the inability of bacteria normally present in conventional treatment systems to degrade greases and fats at an acceptable rate, the treatment of wastewater containing such has been a problem in the past.

With the increasing concern as to minimization of the problems arising from pollution, biological processes utilizing microorganisms are being industrially municipally and domestically employed in an increasing amount, and a large amount of activity in research and development is occurring presently to develop new microbial strains capable of use in wastewater treatment both industrially, municipally and domestically. Even with this increased activity in investigating and developing strains of microorganisms to solve particular waste removal problems, a sufficiently acceptable solution to the problem of removing greases and fats which are present in wastewater from domestic, municipal and industrial sources has not yet been developed.

SUMMARY OF THE INVENTION

Accordingly, an object of this invention is to provide a method whereby oleaginous materials present in domestic, municipal and industrial wastewaters can be removed.

Another object of this invention is to provide a biological method for treatment of industrial, municipal and domestic wastewaters to not only remove biodegradable organic matter therefrom but to specifically reduce the level of or remove oleaginous materials therefrom.

A further object of this invention is to provide a biological treating process for removal of oleaginous materials from industrial, municipal and domestic waste waters using a novel mutant of *Pseudomonas aeruginosa*.

An even further object of this invention is to provide a biological treatment method for industrial, municipal and domestic wastewater to remove oleaginous materials therefrom and render such suitable for discharge into the biosphere, thereby minimizing problems of pollution.

A further object of this invention is to provide a treatment for industrial, municipal and domestic wastewaters whereby a combination of microorganisms acting synergistically with respect to oleaginous materials, particularly those which are of animal origin or which contain those of animal origin, is employed.

An additional object of this invention is to provide a novel combination of microorganisms which act synergistically to degrade oleaginous materials in industrial, municipal and domestic wastewaters.

In one embodiment of this invention, this invention provides a process for removing oleaginous material from wastewater comprising treating wastewater containing oleaginous material with a microbial combination of:

(a) a microorganism of the strain *Pseudomonas aeruginosa* mutant $SGRR_2$; and

(b) at least one of:

(i) a microorganism of the genus Bacillus; and

(ii) a microorganism of the genus Pseudomonas other than the strain *Pseudomonas aeruginosa* mutant $SGRR_2$.

In another embodiment of this invention, this invention provides a microbial combination synergistically acting with respect to degradation of oleaginous material, the microbial combination comprising:

(a) a microorganism of the strain *Pseudomonas aeruginosa* mutant $SGRR_2$; and

(b) at least one of:

(i) a microorganism of the genus Bacillus; and

(ii) a microorganism of the genus Pseudomonas other than the strain *Pseudomonas aeruginosa* mutant $SGRR_2$.

DETAILED DESCRIPTION OF THE INVENTION

The novel mutant of the species *Pseudomonas aeruginosa* $SGRR_2$ was produced by mutation of a parent strain of *Pseudomonas aeruginosa* designated

Pseudomonas aeruginosa HCP (hereinafter "parent strain") isolated from the soil in Salem, Va.

This *Pseudomonas aeruginosa* $SGRR_2$ (hereinafter "mutant strain") has been found, when used in combination with microorganisms of the genus Bacillus and microor-

ganisms of the genus Pseudomonas other than this mutant strain, to be capable of degrading oleaginous materials and has the characteristics described below.

Pseudomonas aeruginosa $SGRR_2$

The cells of the mutant strain are gram-negative rods. The cells are motile and in culture, the cells are straight, not curved rods where less than about 1% of the cells exist in the form of long filaments of greater than five cell-units long. On Kings Medium A (described in E. O. King et al., *J. Lab. & Clin. Med.*, Volume 44, No, 2, page 303 (1954), and on Difco BACTO-Antibiotic Medium No. 3 (trade name produced by Difco Laboratories), a blue-green pyocyanin pigment is formed which diffuses into the surrounding medium. A characteristic grape-like odor is given off by cultures of *Pseudomonas aeruginosa* mutant $SGRR_2$ on both complex media, such as nutrient broth and nutrient agar, and on minimal salts-based media containing a carbon source, such as glucose. The mutant strain is incapable of utilizing acetate as a sole carbon source.

The mutant strain is an obligate aerobe, except in the presence of nitrate. On metabolism in the presence of nitrate, the strain produces nitrate reductase.

The cells of the mutant strain are incapable of accumulating poly-β-hydroxybutyric acid granules even though DL-hydroxybutyrate serves as a sole carbon source.

A preferred growth temperature range is about 20°-41° C., with optimal growth occurring at 37° C. No growth is observed in ten days at 14° C. The mutant strain is capable of growth on a glucose-containing minimal salts medium containing ammonium ion as the nitrogen source. The mutant strain has either only a small or absent requirement for trace metals because it is capable of growth in medium lacking magnesium, manganese, zinc, cobalt and iron which has been made with distilled deionized water. Thus the mutant strain does not appear to require any growth factor or vitamin supplement. The strain displays arginine dihydrolase activity and is capable of gelatin hydrolysis.

Pseudomonas aeruginosa HCP

The parent strain *Pseudomonas aeruginosa* HCP is a gram-negative, non-spore-forming rod. The cells are straight rods which have a single-polar flagellum, and the cells are motile. In culture, approximately 1% of the cells exist in the form of long filaments of greater than five cell units long. On Kings Medium A and on Difco BACTO-Antibiotic Medium 3, solidified with agar at temperatures from 20°-40° C., a blue-green diffusible pigment is formed. A characteristic grape-like odor is given off by cultures of *Pseudomonas aeruginosa* HCP on both complex media, such as nutrient broth and nutrient agar, and on minimal salts-based media containing a carbon source, such as glucose.

The parent strain *Pseudomonas aeruginosa* HCP is capable of growing on either a glucose or acetate containing minimal salts medium (Roy Curtiss, III, *J. Bact.*, 89 28-40 (1965)) containing ammonium ion as a nitrogen source, thus demonstrating the strain does not appear to require any growth factor or vitamin supplement.

The parent strain is an obligate aerobe, although growth is possible anaerobically in the presence of nitrate, in which case a gas is formed. On metabolism in the presence of nitrate, the parent strain produces nitrate reductase.

The cells of the parent strain are incapable of accumulating poly-β-hydroxybutyric acid granules even though DL-hydroxybutyrate serves as a sole carbon source.

A preferred growth temperature range is about 20°-41° C., with optimal growth occurring at 37° C. No growth is observed in ten days at 14° C. The strain displays arginine dihydrolase activity and is capable of gelatin hydrolysis.

Other cultural characteristics and colonial morphology or these two *Pseudomonas aeruginosa* strains are shown in Tables 1-6 below.

In the following tables, *Pseudomonas aeruginosa* strain PAO (ATCC 13525) was employed as a known type strain for characterization purposes.

TABLE 1

MICROSCOPIC MORPHOLOGY

CHARACTERISTIC	STRAIN: *PSEUDOMONAS AERUGINOSA***	HCP	$SGRR_2$
Cell Size*			
Length	1.5-3.0	1.5-3.0	1.5-3.0
Width	0.5-0.8	0.5-0.8	0.5-0.8
Gram Reaction	Negative rod	Negative rod	Negative rod

*Wet mounts of ten-hour cultures (late exponential phase) viewed under phase contrast (1000×). Sizes given in micrometers.
**Data from Bergey's Manual of Determinative Bacteriology, 8th Ed., The Williams & Wilkins Co., Baltimore (1974).

TABLE 2

COLONIAL CHARACTERISTICS OF *PSEUDOMONAS AERUGINOSA* HCP AND $SGRR_2$ (After 48 Hours At 35° C.)

Pseudomonas aeruginosa - HCP

Plate Count Agar

Colonies are circular, flat and have a rough surface. Their edge is undulate and they are 5-10 mm in diamteter. They are transparent and white in color. No pigment is produced.

Nutrient Agar

Colonies are slightly irregular, flat and have a wrinkled surface. Their edge is undulate and they are 4-7 mm in diameter. They are transparent and white in color. No pigment is produced.

Hektoen Enteric Agar

Colonies are slightly irregular, slightly convex with a wrinkled surface. They have an undulate edge and they are from 4-6 mm in diameter. They are transparent and green in color. No pigment is produced.

Pseudosel Agar

Circular, convex colonies are smooth and have an entire edge. They are slightly opaque, white and are 1-1.5 mm in diameter. A fluorescent green pigment is produced.

Trypticase Soy Agar

Slightly irregular colonies are flat and have a wrinkled edge. Their edge is undulate. They are white in color and are 3-6 mm in diameter. They are slightly opaque. A fluorescent yellow pigment is produced.

Pseudomonas aeruginosa - $SGGR_2$

Plate Count Agar

Colonies are slightly irregular, flat and have a rough surface. Their edge is undulate and they are transparent with an opaque center. They measure 3-5 mm in diameter. They are white and produce a diffusible blue-green pigment.

Nutrient Agar

Colonies are irregular, flat and have a wrinkled surface. They are transparent with an opaque center, are 4-5 mm in diameter and have an undulate edge. The colonies are white and produce a diffusible green pigment.

Hektoen Enteric Agar

TABLE 2-continued

COLONIAL CHARACTERISTICS OF *PSEUDOMONAS AERUGINOSA* HCP AND $SGRR_2$ (After 48 Hours At 35° C.)
Slightly irregular colonies are slightly undulate, have a wrinkled surface and are transparent. They are 6–7 mm in diameter, have an undulate edge, are green in color, and produce no pigment.
Pseudosel Agar
Circular, flat colonies with a rough surface have an entire edge. They are 3–6 mm in diameter. They are transparent and colorless, and produce a diffusible blue pigment.
Trypticase Soy Agar
Colonies are circular, flat, transparent, have an undulate edge and have a rough surface. They are 5–7 mm in diameter. They are white, and produce a fluorescent diffusible green pigment.

NOTE:
Plate Count Agar and Hektoen Enteric Agar are products of Difco Laboratories. Pseudosel Agar, Nutrient Agar and Trypticase Soy Agar are products of Baltimore Biological Laboratories.

TABLE 3

UTILIZATION OF CARBON-CONTAINING COMPOUNDS FOR GROWTH

	GROWTH RESPONSE**		
COMPOUND*	*PSEUDOMONAS AERUGINOSA*	HCP	$SGRR_2$
Carbohydrates (& Sugar Derivatives)			
α - Cellulose	+	+	+
L - Arabinose		−	−
D - Ribose	−	−	−
D - Glucose		+	+
Sucrose		−	−
Trehalose		−	−
D - Cellobiose	−	−	−
Xylose	−	−	−
Organic Acids			
Acetate		+	−
Propionate	−	−	−
Butyrate		+	+
Isobutyrate	−	−	−
Valerate	−	−	−
Caproate	−	−	−
Heptanoate	−	−	−
Caprate	−	+	+
Stearate	+	+	−
Dicarboxylic Acids			
Maleate	+	+	+
Malonate	−	−	−
Succinate	−	−	−
Glutarate	−	−	−
Saccharate		−	−
Hydroxyacids			
L - Malate	−	−	−
DLβ - Hydroxybutyrate		+	+
DL - Lactate	−	−	−
DL - Glycerate	+	+	+
Miscellaneous Organic Acids			
Citrate	−	−	−
α - Ketoglutarate	−	−	−
Pyruvate	+	+	+
Polyhydric Alcohols And Glycols			
Mannitol	+	+	+
Glycerol	+	+	+
Propyleneglycol		+	+
m - Inositol		−	−
Sorbitol		−	−
Alcohols			
Ethanol		+	+
n - Propanol	+	−	−
n - Butanol	±	+	+
Non-Nitrogenous Aromatic And Other			

TABLE 3-continued

UTILIZATION OF CARBON-CONTAINING COMPOUNDS FOR GROWTH

	GROWTH RESPONSE**		
COMPOUND*	*PSEUDOMONAS AERUGINOSA*	HCP	$SGRR_2$
Cyclic Compounds			
Benzoate	−	−	−
Aliphatic Amino Acids			
Lα - Alanine	+	−	−
Dα - Alanine	−	−	−
β - Alanine		+	+
L - Leucine	−	+	+
L - Aspartate	+	+	+
L - Glutamate	+	+	+
L - Lysine	+	−	+
DL - Arginine		+	+
L - Valine		−	−
Glycine		−	−
Asparagine	+	+	+
Amino Acids And Related Compounds Containing A Ring Structure			
L - Histidine	+	−	+
L - Proline	−	+	+
L - Tyrosine	−	−	−
Miscellaneous Nitrogenous Compounds			
Betaine	+	+	+
Sarcosine	−	−	−
Acetamide	+	+	+
Glucosamine	−	−	−
Detergents***			
Igepal CO 520 (2000 mg/l)	±	−	−
Igepal CO 610 (2000 mg/l)	±	−	−
Igepal CO 660 (2000 mg/l)	±	−	−

*Compound added at 0.5% to minimal salts medium (Curtiss (1965)).
**+ indicates growth greater than blank;
− indicates growth less than that of blank;
± indicates growth approximately equal to blank or weak growth, after 7 days at 30° C.
***Trade name for a non-ionic nonylphenol-ethylene oxide condensate produced by GAF.

TABLE 4

UTILIZATION OF NITROGENOUS COMPOUNDS AS SOLE NITROGEN SOURCE

	GROWTH RESPONSE**		
COMPOUND*	*PSEUDOMONAS AERUGINOSA*	HCP	$SGRR_2$
NH_4Cl	−	−	−
KNO_3	−	−	−
L - Glutamate	+	+	+
L - Aspartate	−	−	−
L - Alanine	−	−	+

*Compound added at 0.5 g/100 ml to minimal salts medium (Curtiss (1965) but without NH_4Cl and NH_4NO_3) consisting of 0.5 g of D-glucose/100 ml.
**+ indicates growth greater than blank;
− indicates growth less than that of blank;
± indicates growth approximately equal to blank or weak growth, after 7 days at 30° C.

TABLE 5

CULTURE GROWTH IN PRESENCE OF HEAVY METALS

		STRAIN RESPONSE**		
HEAVY METAL*	CONCENTRATION	*PSEUDOMONAS AERUGINOSA*	HCP	$SGRR_2$
$HgSO_4$	$2 \times 10^{-3}M$	−	−	−
	$10^{-3}M$	−	−	−
	$10^{-4}M$	−	−	+
	$10^{-5}M$	+	+	+
$CdCl_2$	$2 \times 10^{-3}M$	−	−	−
	$10^{-3}M$	−	−	−

TABLE 5-continued

CULTURE GROWTH IN PRESENCE OF HEAVY METALS				
		STRAIN RESPONSE**		
HEAVY METAL*	CONCENTRATION	*PSEUDOMONAS AERUGINOSA*	HCP	$SGRR_2$
	10^{-4}M	+	−	+
	10^{-5}M	+	−	+
$CoCl_2$	2×10^{-3}M	−	−	+
	10^{-3}M	−	−	+
	10^{-4}N	+	+	+
	10^{-5}M	+	+	+
$AgSO_4$	2×10^{-3}M	−	−	−
	10^{-3}M	−	−	−
	10^{-4}M	−	−	−
	10^{-5}M	−	−	−
Na_2HAsO_4	2×10^{-3}M	−	+	+
	10^{-3}M	+	+	+
	10^{-4}M	+	+	+
	10^{-5}M	+	+	+

*Heavy metal added to D-glucose containing (0.5%) minimal salts medium (Curtiss (1965)).
**Growth response scored: + indicates growth (no inhibition); − indicates no growth (inhibition).

TABLE 6

RESISTANCE OF CULTURES TO ANTIBIOTICS		
	STRAIN RESPONSE	
ANTIBIOTIC	HCP	$SGRR_2$
Ampicillin	R*	R
Carbenicillin	S	I
Cephalothin	R	R
Chloramphenicol	R	R
Coly-mycin	S	S
Gentamicin	S	S
Kanamycin	R	R
Mandol	R	R
Streptomycin	R	R
Tobramycin	S	S
Tetracycline	R	R
Amikacin	S	S

*Growth response on Pfizer Antimicrobial Suspectibility Disks; Pfizer, Inc. scored: S = sensitive to antibiotic; R = resistant to antibiotic; and I = intermediate.

On the basis of the morphological, cultural and physiological characteristics set forth above, the mutant strain has been identified as a member of the species *Pseudomonas aeroginosa* and has been designated herein as *Pseudomonas aeruginosa* $SGRR_2$. A culture of the strain has been deposited in the American Type Culture Collection and has received an accession number, ATCC-31480.

Further, on the basis of the morphological, cultural and physiological characteristics set forth above, the parent strain from which the mutant strain was developed has been identified as a member of the species *Pseudomonas aeruginosa* and has been designed herein as *Pseudomonas aeruginosa* HCP. A culture of the strain has been deposited in the American Type Culture Collection and has received an accession number, ATCC-31479.

As indicated above, the parent strain HCP, from which the mutant strain, $SGRR_2$, was developed, was isolated and was the mutated in the following manner. The mutation was carried out in a bench-top biotower which basically was a trickling filter. The biotower comprised a reservoir for a liquid and a column containing Pall rings of a plastic resin, one end of which column was placed just above the liquid in the reservoir. A pump was submerged in the liquid reservoir for recycling liquid through a tube to the top of the column for dispersion of the liquid down through the Pall ring packing.

The biotower was first pre-conditioned by forming a slime layer of the HCP culture on the Pall rings using a 3-liter volume of deionized water containing 2% whey, 0.5% disodium phosphate and 0.1% $(NH_4)_2SO_4$ inoculated with the HCP culture. This was allowed to circulate over the tower for 2 days at room temperature (about 20°-30° C.) until a heavy slime layer formed on the rings. Then 200 ppm Santophen-1 (a chlorinated phenol-type disinfectant, trade name produced by Monsanto) was added along with sufficient nonyl phenol-type surface active agent (Igepal CO 660, trade name produced by GAF) to disperse the Santophen-1.

Recycling of the liquid through the biotower was allowed to proceed for another 2 days at which time 0.2% 8-azaguanine, as a strong chemical mutagent, was added. Recycling of the liquid through the biotower was continued for another 72 hours at which time the reservoir containing the 3 liters of liquid as described above was poured out. The column was then rinsed by recycling with fresh deionized water and again filled to the 3-liter level and 500 ppm of Santophen-1 added.

Three different colony types of microorganisms that grew through this were isolated after 48 hours and set aside, and one of these was designated *Pseudomonas aeruginosa* $SGRR_2$. As will be demonstrated in greater detail in the Examples given hearinafter, this mutant was tested against oleaginous materials and found to be substantially non-active against oleaginous materials in wastewater when used alone, as was the parent strain *Pseudomonas aeruginosa* HCP, but synergistically active in degrading oleaginous materials when used in combination with the parent strain. Further, it was found that when the $SGRR_2$ mutant strain was used in combination with microorganisms of the genus Bacillus, such as *Bacillus subtilis,* also substantially not active alone against oleaginous materials, the combination was synergistically active against oleaginous materials.

Thus, it has been found that when the mutant strain *Pseudomonas aeruginosa* $SGRR_2$, including variants thereof, is employed in combination with the parent strain, *Pseudomonas aeruginosa* HCP, including variants thereof, or with a bacterium of the genus Bacillus, an extremely high, synergistic activity on degradation of oleaginous materials in wastewater is found.

While not desiring to be bound, and while the reasons for this synergistic activity at the present time are not completely understood, it is surmised that the presence of the *Pseudomonas aeruginosa* $SGRR_2$ in combination with another microorganism of the genus Pseudomonas or with a microorganism of the genus Bacillus that the metabolic path in degrading oleaginous materials of one organism complements or supplements that of the other organism present in combination therewith, resulting in this high, unexpected synergistic activity.

The microbial combination of (a) the *Pseudomonas aeruginosa* $SGRR_2$ and (b) the other organism of the genus Bacillus or the genus Pseudomonas (other than *Pseudomonas aeruginosa* $SGRR_2$) can be employed in cell count proportions ranging from about 1:99 to about 99:1 of (a) to (b) to achieve the objects of this invention.

The microbial combination employed in this invention can be cultured in wastewater containing the oleaginous material either using a batch process, a semi-continuous process or a continuous process, and such a microorganism combination is cultured for a time sufficient to degrade the oleaginous materials present in the wastewater and remove them or break them down into components capable of being degraded by other organ-

isms normally found in biological wastewater treatment systems.

The microbial combination of this invention can be employed in ion exchange resin treatment systems, in trickling filter systems, in activated sludge treatment systems, in outdoor lagoons or pools, etc. Basically, all that is necessary is for the microbial combination to be placed in a situation of contact with wastewater containing the oleaginous material. In order to degrade the oleaginous material present in the wastewater, the microbial combination can be cultured at conditions of about 15° C. to about 42° C., preferably about 20° C. to about 38° C. Desirably, the pH is maintained in a range of about 5.5 to about 8.5, preferably 6.5 to 8.0. Control of the pH can be by monitoring of the system and an addition of appropriate pH adjusting materials to achieve this pH range.

The culturing is conducted basically under aerobic conditions of a dissolved oxygen concentration of about 2 ppm or more, preferably about 5 ppm or more. These conditions can be simply achieved in any manner conventional in the art and appropriate to the treatment system design being employed. For example, air can be bubbled into the system, the system can be agitated, a trickling system can be employed, etc.

Normally, the wastewater to be subjected to the process of this invention will contain sufficient nitrogen and phosphorus for culturing without the need for any additional source of nitrogen or phosphorus being added. However, in the event the wastewater is deficient in these two components, suitable available nitrogen sources, such as ammonia or an ammonium salt, e.g., ammonium sulfate, can be added to achieve an available nitrogen content of at least about 10 ppm or more per 100 BOD_5. Similarly, phosphorus can be supplemented, if necessary, by addition of materials such as orthophosphates, e.g., sodium phosphate, to achieve a phosphorus level in the wastewater of about 1 ppm or more per 100 BOD_5. In general, the treatment is conducted for a sufficient time to achieve the reduction in oleaginous material content desired and, in general, about 3 hours to about 1 week or longer, although this will depend upon the temperature of culturing, the volume to be treated and other factors, has been found to be suitable.

In the above manner, difficulty degradable oleaginous materials, such as those of animal origin or those containing oleaginous materials of animal origin, as well as other organic compounds which might be present in such wastewater streams, can be advantageously treated to provide treated wastewater suitable for discharge after any additional conventional processing such as settling, chlorination, etc. into rivers and streams.

The microbial combination of this invention employed in the process of this invention has been found to be extremely advantageous in the treatment of wastewater containing oleaginous materials. The microbial combination employed in this invention is particularly advantageous since such is resistant to shock loads due to the presence of high levels of toxic materials, such as heavy metals, and organic solvents, such as aromatic and aliphatic solvents, pesticides, chlorinated compounds, disinfectants, phenolics, thiocyanates, etc., which would basically poison a conventional biological treatment system and disrupt the metabolic pathway of the conventional organisms utilized. The microbial combination of this invention is resistant to such shock loads, and based on information to date, it is believed that many industrial wastewater systems from a variety of different types of industrial installations can be treated using the microbial combination of this invention.

Suitable examples of other organisms of the genus Pseudomonas, other than the *Pseudomonas aeruginosa* $SGRR_2$ mutant strain, which can be employed in the combination of this invention include those of the alcaligenes group, such as *Pseudomonas alcaligenes,* and those of the fluorescent group, such as *Pseudomonas fluorescens, Pseudomonas putida,* and other *Pseudomonas aeruginosa* strains.

Additionally, suitable microorganisms of the genus Bacillus which can be employed in combination with the *Pseudomonas aeruginosa* $SGRR_2$ mutant strain in this invention include *Bacillus subtilis, Bacillus licheniformis, Bacillus cereus, Bacillus thuringiensis, Bacillus megaterium, Bacillus circulans, Bacillus coagulans, Bacillus brevis, Bacillus sphaericus, Bacillus fastidiosus,* etc.

As can be seen from an examination of the results set forth in the Examples below, the microbial combination of this invention utilized in the process of this invention results in the ability to obtain approximately a 75-fold increase over that obtained with the use of Bacillus strains alone, and approximately a 4-fold increase over the results obtained where the *Pseudomonas aeruginosa* $SGRR_2$ mutant strain is employed alone or where the parent *Pseudomonas aeruginosa* HCP;, from which the mutant strain was developed, is used alone. This 75-fold and 4-fold increase in the use of the microbial combination of this invention is highly unexpected in view of the performance of these microorganisms separately and thus provides the ability to effectively degrade and remove oleaginous materials from wastewater, thereby providing a solution to a wastewater treatment problem presently existing.

The term oleaginous material as employed herein basically includes any material of an oily, fatty or greasy nature of animal origin or containing such of animal origin, with specific examples including oils, fats and greases, such as lard, beef tallow, butter, chicken fat, and other animal fats.

The following Examples are given to illustrate the present invention in greater detail but are given merely for the purposes of exemplification and are not to be construed as limiting the scope of the present invention. Unless otherwise indicated herein, all parts, percents, ratios and the like are by weight.

EXAMPLE 1

One gram of lard (commercially available under the trade name "Valleydale Lard," containing BHT, BHA and propyl gallate), as an oleaginous material of animal origin, was placed in 100 ml of a basic salt solution having the composition set forth below in cotton-stoppered 250 ml Erlenmeyer flasks.

Basic Salt Solution	
NH_4Cl	5 g/l
Na_2SO_4	2 g/l
KH_2PO_3	3 g/l
$MgSO_4$*	1 g/l
NH_4NO_3	1 g/l
K_2HPO_4	9 g/l

*Added after sterilization and cooling.

The flasks were sterilized, cooled and inoculated with one loop full of each of the microorganisms set forth below, and the flasks and contents were shaken for 5 days on a rotary shaker.

The results were analyzed by comparing flasks containing the microorganisms with an uninoculated control flask prepared and treated in the same manner as described above. The analysis was of the oil and grease amount present using a gravametric determination of the dried residue remaining after extraction of each flask, with 1,1,2-trichloro-1,2,2-trifluoroethane (based on *Standard Methods for the Examination of Waters and Wastewaters*, 14th Ed., page 513, Water Pollution Control Federation (1975)).

The results set forth in Table 7 below were obtained.

TABLE 7

CULTURE	OIL & GREASE AMOUNT (GRAMS)	% REDUCTION*
Control (none)	0.9732	—
Comparison *Bacillus* Microorganism Combination	0.9637	0.97
Pseudomonas aeruginosa HCP	0.7907	18.75
Pseudomonas aeruginosa $SGRR_2$	0.8279	14.93
Pseudomonas aeruginosa $SGRR_2$ + *Pseudomonas aeruginosa* HCP	0.3186	67.26
Pseudomonas aeruginosa $SGRR_2$ + Comparison *Bacillus* Microorganism Combination	0.2390	75.44

*% Reduction is based on control flask.
Comparison *Bacillus* Microorganism Combination comprised an equal mixture by volume of *Bacillus subtilis* (strain producing mainly protease), *Bacillus subtilis* (strain producing mainly amylase), *Bacillus cereus* and *Bacillus circulans* (strain producing mainly cellulase).

COMPARISON EXAMPLE

The procedures described in Example 1 were repeated on a vegetable oil (commercially available under the trade name "Kroger Pure Vegetable Oil", a partially saturated soybean oil with oxystearin added and BHT, BHA and methyl silicone), as an oleaginous material of vegetable origin, to demonstrate the advantageous results obtained in this invention in degrading oleaginous material of animal origin or containing those of animal origin.

The results obtained are set forth in Table 8 below.

TABLE 8

CULTURE	OIL & GREASE AMOUNT (GRAMS)	% REDUCTION*
Control (none)	0.9887	—
Comparison *Bacillus* Microorganism Combination	0.5998	39.33
Pseudomonas aeruginosa HCP	0.0697	92.95
Pseudomonas aeruginosa $SGRR_2$	0.1739	82.41
Pseudomonas aeruginosa $SGRR_2$ + *Pseudomonas aeruginosa* HCP	0.0610	93.31
Pseudomonas aeruginosa $SGRR_2$ + Comparison *Bacillus* Microorganism Combination	0.2640	73.29

*% Reduction is based on control flask.
Comparison *Bacillus* Microorganism Combination comprised an equal mixture by volume of *Bacillus subtilis* (strain producing mainly protease), *Bacillus subtilis* (strain producing mainly amylase), *Bacillus cereus* and *Bacillus circulans* (strain producing mainly cellulase).

EXAMPLE 2

In order to further demonstrate the synergistic effect obtained with the microbial combination of this invention in utilization of such in combination with other microorganisms outside the scope of this invention, the following procedures were conducted.

Caprate, a known fatty acid component of oleaginous materials, was employed, and degradation thereof in an agar-based substrate was evaluated. The degradation of the caprate in the agar base is exhibited by a clearing of the agar around the microorganism colony growth. Thus, a clear zone around a disk inoculated with a microorganism and placed on the caprate-containing agar base is formed, and by comparing the sizes of such zones made by various microorganisms or combinations thereof, the effectiveness of the microorganisms in degrading caprate can be thereby determined.

The procedures followed in this comparison involved suspending 24-hour cultures of each of the microorganisms set forth in Table 9 below in sterile physiological saline. All cultures, after suspension, had approximately equal optical densities. The suspensions of the microorganisms were mixed in the concentrations set forth in Table 9 below and absorbed on a paper disk of sterile filter paper having a diameter of 13 mm. The disks were then placed on the agar base containing 0.5% caprate plus minimal salts (Curtiss (1965)), and after 72 hours of incubation at 35° C., the zones of clearing were measured.

The results set forth in Table 9 below were obtained.

TABLE 9

PERCENT OF CULTURE IN COMBINATION			
HCP	$SGRR_2$	CBMC	ZONE OF CLEARING (mm)
1	99	—	21
10	90	—	22
30	70	—	21.6
60	40	—	22
90	10	—	21
99	1	—	20
0.5	0.5	99	19.5
5	5	90	24
15	15	70	23.5
30	30	40	25
45	45	10	20
49.5	49.5	1	21
—	1	99	21.5
—	10	90	24
—	30	70	23.75
—	60	40	22.5
—	90	10	20.5
—	99	1	21
1	—	99	21
10	—	90	22
30	—	70	22
60	—	40	22.5
90	—	10	23.25
99	—	1	23.5

TABLE 9-continued

PERCENT OF CULTURE IN COMBINATION					ZONE OF CLEARING
HCP + $SGRR_2$*	BSP	BSA	BC	BCC	(mm)
—	100	—	—	—	0
—	—	100	—	—	0
—	—	—	100	—	0
—	—	—	—	100	0
50	50	—	—	—	22
50	—	50	—	—	22
50	—	—	50	—	21.25
50	—	—	—	50	22.75
33	33	33	—	—	22.75
33	33	—	33	—	23
33	33	—	—	33	21.5
33	—	33	33	—	19.5
33	—	33	—	33	21.5
33	—	—	33	33	23
25	25	25	25	—	21.5
25	25	25	—	25	22
25	25	—	25	25	20.25
25	—	25	25	25	22
25	25	25	25	25	19

*50% by volume each of HCP and $SGRR_2$.
NOTE: Zone of Clearing measured in millimeters, and result presented is an average of two different diameter measurements of the zone.
HCP = *Pseudomonas aeruginosa* HCP.
$SGRR_2$ = *Pseudomonas aeruginosa* $SGRR_2$.
BSP = *Bacillus subtilis* (strain mainly producing protease).BSA = *Bacillus subtilis* (strain mainly producing amylase).
BC = *Bacillus cereus.*
BCC = *Bacillus circulans* (strain mainly producing amylase).
CBMC = Comparison *Bacillus* Microorganism Combination.

EXAMPLE 3

In this evaluation of the microbial combination of this invention, the waste treatment system of a large poultry processing plant through which 75,000 chickens were processed per day was employed. The wastewater treatment system comprised nine aerated lagoons of which the pH of the wastewater was monitored and adjusted to a pH of 6.5–7.5 by addition of bicarbonate as needed.

In this evaluation, a microorganism combination in dry form comprising 25% *Pseudomonas aeruginosa* HCP, 20% *Pseudomonas aeruginosa* $SGRR_2$, 20% of another *Pseudomonas aeruginosa* of substantially no activity against oleaginous materials and 25% of an equal mixture of *Bacillus subtilis* (strain mainly producing protease), *Bacillus subtilis* (strain mainly producing amylase), *Bacillus cereus* and *Bacillus circulans* (strain mainly producing cellulase), along with 10% unidentified microorganisms was employed.

Effluent wastewater was passed through the treatment system in an amount of 500,000 gallons per day, and the dry microorganism combination was placed in the first lagoon for wash through into the subsequent lagoons in the system. The dry microorganism culture was added in the following amounts:

50 pounds per day for 2 days
25 pounds per day for 3 days
10 pounds per day for 7 days
5 pounds per day for 7 days
3 pounds per day for each day thereafter

During the test, the dissolved oxygen content, due to the aeration, was maintained above 2 ppm.

Samples were periodically obtained from the fourth lagoon and analyzed for chemical oxygen demand, biological oxygen demand, suspended solids and the presence of oil and grease, with the results set forth in Table 10 below being obtained. The day 0 results represent a 3-month average of the treatment system characteristics prior to the evaluation.

TABLE 10

	EVALUATION			
DAY	COD	BOD	SUSPENDED SOLIDS	OIL & GREASE
0	2620	260	401	104
8	1600	132	485	20
15	680	105	368	31
18	450	109	330	18
21	460	120	346	34

It was also found that no salmonella were present in the treated wastewater and the level of fecal coliforms was considerably reduced, the presence of these organisms previously being a problem.

While the invention has been described in detail and with respect to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

What is claimed is:

1. A microbial synergist combination comprising:

(a) a microorganism of the strain *Pseudomonas aeruginosa* $SGRR_2$ (ATCC-31480); and

(b) at least one of:

(i) a microorganism of the genus Bacillus; and

(ii) a microorganism of the genus Pseudomonas other than said strain *Pseudomonas aeruginosa* $SGRR_2$, said combination of microorganisms (a) and (b) synergistically acting to utilize oleaginous materials of animal origin or containing the same as a source of assimilable carbon and degrade said oleaginous materials of animal origin or containing the same.

2. The combination of claim **1**, wherein said microorganism of the genus Bacillus is *Bacillus subtilis, Bacillus licheniformis, Bacillus cereus, Bacillus thuringiensis, Bacillus megaterium, Bacillus circulans, Bacillus coagulans, Bacillus brevis, Bacillus sphaericus* or *Bacillus fastidiosus.*

3. The combination of claim **1**, wherein said microorganism of the genus Bacillus is *Bacillus subtilis, Bacillus cereus* or *Bacillus circulans.*

4. The combination of claim **1**, wherein said microorganism of the genus Bacillus is *Bacillus subtilis.*

5. The combination of claim **1**, wherein said microorganism of the genus Pseudomonas is *Pseudomonas aeruginosa* HCP (ATCC-31479), *Pseudomonas aeruginosa* other than said *Pseudomonas aeruginosa* HCP, *Pseudomonas alcaligenes* or

Pseudomonas putida.

6. The combination of claim **1**, wherein said microorganism of the genus Pseudomonas is *Pseudomonas aeruginosa* HCP (ATCC-31479).

* * * * *

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EXAMINER	
LEVY, NEIL S	

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Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

EXHIBIT

tabbies

B

Office Action Summary	Application No.	Applicant(s)
	10/040,046	TAYLOR, JOHN B.
	Examiner	Art Unit
	NEIL LEVY	1615

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *23 March 2010*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-14* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-14* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Defective Reissue Oath/Declaration, 37 CFR 1.175

This reissue was filed without a reissue oath.

The reissue oath/declaration filed 10/16/07 with this application is defective (see 37 CFR 1.175 and MPEP § 1414) because it is an "Assignee Oath" and not an "Inventor Oath". A broadening reissue application must be applied for by all of the inventors (patentees),that is, the original reissue oath or declaration must be signed by all of the inventors. See also MPEP § 1414.

Please consider using PTO/SB/51 (10-05) found on the U.S. PTO website.

Consent of Assignee to Reissue Lacking

This application is objected to under 37 CFR 1.172(a) as lacking the written consent of all assignees owning an undivided interest in the patent. The consent of the assignee must be in compliance with 37 CFR 1.172. See MPEP § 1410.01. A proper assent of the assignee in compliance with 37 CFR 1.172 and 3.73 is required in reply to this Office action.

Rejection, Defective Reissue Oath or Declaration

Claims 1-14 are rejected as being based upon a defective reissue [2] under 35 U.S.C. 251 as set forth above. See 37 CFR 1.175

Rejection, 35 U.S.C. 251, Broadened Claims Filed by Assignee

Claims 3-14 are rejected under 35 U.S.C. 251 as being improperly broadened in a reissue application made and sworn to by the assignee and not the patentee. Here the added method claims are broader than the issued composition claims 1-2. A claim is broader in scope than the original claims if it contains within its scope any conceivable product or process which would not have infringed the original patent. A claim is broadened if it is broader in any one respect even though it may be narrower in other respects

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 4& 10 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

These claims are redundant. Claim 1 requires 0.25 to 5%.

Claim1 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

If the second formula is, as claimed, a salt, then one of R1 and R3 must be K. This is not claimed. Please amend.

If not amended, applicant is using "salt" in a non-accepted manner.

Claim 1 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicant changed alkinyl to alkynyl. The first should be bracketed; the second, underlined.

Claim 2 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in e relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

There is no basis for a pH and a minimum of 20 nM. The new matter should be removed.

Claims 6-8 and 12-14 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

.

No preparation is disclosed as is claimed. Item C at Column 4 of the patent states that solutions of the compounds are mixed. No KOH is evident, as the solution of the compounds at A. and B. are the claimed phosphonates and water, and phosphates and water.

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Claim Rejections - 35 USC § 102

Claims 1 is rejected under 35 U.S.C. 102(b) as being anticipated by Spraker-4350770.

(a) Spraker discloses a basic salt solution in Example I (column68) which defines a composition that fully meets the limitations of the instant claims.

The monobasic potassium phosphate (K_2HPO_4) is present at a concentration of 52 mM. This same basic salt solution also contains dibasic phosphite (KH_2PO_3) at a concentration of 25 mM.Theseconcentrations of potassium phosphite and potassium phosphate fall within the range of "about 20 ml~to about 5% v/v" in claim1. The open language of "comprising" permits the additional components of the Spraker

compositions which include ammonium chloride, ammonium sulfate, magnesium sulfate, and ammonium nitrate. The intended use as a fertilizer is interpreted to mean that the fertilizer cannot be toxic to plants and stimulates plant growth. The growth stimulating activities are considered to be an inherent property of any composition containing all of the required ingredients at the stated concentrations.

See In re Fitzgerald, 205 USPQ 594 (CCPA 1980). See also In re May, 197 USPQ 601,607 (CCPA 1978).

The instantly claimed fertilizers are anticipated by the Spraker salt solution (col. 10, lines 60 - 68) because the Spraker salt solution contains potassium phosphite and potassium phosphate at a concentration of between about 20mM and 5% vol/vol. The fungicidal activity and plant growth stimulating activities are deemed to be an inherent characteristic of any composition which meets the concentration limits of phosphite and phosphate, the molar ratio of phosphite to phosphate in the claims, and which lacks any toxic effects on plants generally.

Claim Rejections - 35 USC § 103

Claims 1,2, STAND rejected under 35 U.S.C. 103(a) as being unpatentable over Spraker- 4350770.

Response to Arguments

Applicant's arguments filed 3/23/2010 have been fully considered but they are not persuasive. Applicant argues SPRAKER is basic, and not for foliar spray fungicidal effects.

Claimed is a composition, its use obvious to test for to determine optimal concentrations.

Example I meets the instant claim 1 requirement. SPRAKER shows pH as low as 5.5-(col.9, lines 13-17) that meets the instant claim 5 to 7 ,and the functional advantage as a foliar spray is not claimed. One in the art would find it obvious to test to determine effective amounts.

Applicant should show the range of concentrations, inclusive of pH 5.5-7.0, would not encompass the instant claimed amounts of at least 20nM.

It would have been well within the skill of one of ordinary skill in the art to have applied the combination of potassium phosphonate salts and potassium phosphate salts to plants with the expectation that said combination would have been effective in promoting the growth of plants. Further, one of ordinary skill in the art would have been motivated to vary the amount or concentration of said salts, including the amounts or concentrations falling within the claimed values, as desired, depending on the host plant and the climate conditions. This would have simply been routine experimentation by the artisan to find the optimal concentration of phosphite and phosphate. Therefore, the

claimed invention, as a whole, would have been prima facie obvious to one of ordinary skill in the art at the time of the invention was made, with adjustment of pH as necessary to maintain a solution of the composition

Claims 1, 6 & 12 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Fenn et al '84 & Dolan et al '88, with evidence exemplified by Barlet-5070083.

Claims 1, ,6& 12 STAND rejected under 35 U.S.C. 103(a) as being unpatentable over Barlet 5070083,Ducret et al 4139616,Horriere et al 5169646, Lovatt 5514200, Vetanovetz et al 53905418 and Smilie et al '89.

Here, too, we find one in the art would be aware of these references & straight forward testing would enable one to achieve desired effects, in accord with standard practice in the horticultural arts. These references, of record, teach the instant Phosphonate salts are well known, art recognized fungicides (Bartlet, Ducret & Horriere) while Lovatt & Vetanovetz likewise teach the Phosphates are well known, art recognized fertilizers. Smillie shows the effectiveness of phosphite salts are enhanced with phosphates (p 924 of Smillie).

It would have been obvious to a person of ordinary skill in the art at the time the invention was made desiring to utilize fungicidal control means on plants, to use any of art recognized means, modified as desired to increase stability, dispersibility, compatability of ingredients, processing ease, decreased toxicity to handlers, increased

toxicity to fungus, & to provide added benefit;fertilizer & fungicide in one application. The particular manner in which the compositions are made is not seen to be of patentable weight. Simple testing would enable one in the art to determine the optimal amounts needed , & is in the purview of the artisan to perform. See KSR V TELEFLEX @ 82 USPQ 2d @ 1385.

The amounts and proportions of each ingredient are result effective parameters chosen to obtain the desired effects. It would have been obvious to vary the form of each ingredient to optimize the effect desired, depending upon the particular species and application method of interest, reduction of toxicity, cost minimization, enhanced, and prolonged, or synergistic effects.

Applicant has not provided any objective evidence of criticality, nonobvious or unexpected results that the combination of the particular ingredients' or concentrations provides any greater or different level of prior art expectation as claimed, and the use of ingredient for the functionality for which they are known to be used is not basis for patentability.

The instant invention provides well known old art recognized compounds, with well known art recognized effects, applied by well known art recognized methods to achieve improved control as is well known in the art.

Applicant's arguments filed 3/23/2010 have been fully considered but they are not persuasive.

Applicant argues the SMILIE reference showed the opposite of the claimed invention, and preponderance of evidence favors phosphate inhibiting the phosphite enhancement. However, examiner finds SMILIE shows one must test the specific strain of plant of concern to determine optimum concentration of phosphate, if any, to add to an intended phosphite.

It would have been well within the skill of one of ordinary skill in the art to have applied the combination of potassium phosphonate salts and potassium phosphate salts to plants with the expectation that said combination would have been effective in promoting the growth of plants and controlling fungal infections. Further, one of ordinary skill in the art would have been motivated to vary the amount or concentration of said salts, including the amounts or concentrations falling within the claimed values, as desired, depending on the susceptibility of the fungus, the extent of disease, the host plant and the climate conditions. This would have simply been routine experimentation by the artisan to find the optimal concentration of phosphite and phosphate. Therefore, the claimed invention, as a whole, would have been prima facie obvious to one of ordinary skill in the art at the time of the invention was made, because every element of the invention has been collectively taught by the combined teachings of the references and clear motivation existed to combine the references.

Claim1,3-5,9-11 are rejected under 35 U.S.C. 103(a) as being unpatentable over Thyzy et al. (4,075,324) in view Reuveni et al. (J. Phytophath- ology, 141 : 337 - 346 1994), Reuveni et al. (Plant Pathology 44:31 - 39, 1995), Dunstan et al. (Physiol. Molec. Plant Path. 36:205 - 220, 1990), the Fenn Dissertation (1996), Walker (Fungic. Nematic. Tests, 1994), and the Fluid Fertilizer Manual (1995).

Thizy et al. disclose fungicidal compositions containing monopotassium phosphite (KH2PO3) and compositions containing potassium phosphite (K2HPO3) at

column 1, lines 52 - 56 and column 2, lines 32 - 36. It is disclosed that the above fungicidal compositions are generally not applied to crops alone, but are applied in combination with other materials, such as support, which can be a mineral material which facilitates the application to the plant and can be solid or fluid (column 8, line 60 - column 9, line 15). It is also disclosed that the above fungicidal compositions can be mixed with other fungicidal, anti-mildew phosphorous derivatives (column 8, lines 44 - 46). It is further disclosed that 0.5 g/L of KH2PO3 (compound 4) or K2HPO3 (compound 5) applied to plants prior to infestation with Plasmopara vitico/a afforded total protection and that 1 g/L of the same (6.3 mM of K2HPO3 and 8.3 mM of KH2PO3) applied after infestation completely stopped the development of mildew on the plant (column 2, lines 31 - 38 and column 6, lines 31 - 68). Finally, Thizy et al. disclose that doses of from 0.01 to 5 g/L are generally suitable but that the doses may vary within wide limits depending on both the virulence of the fungus and upon the climate conditions (column 8, lines 55 - 59). 5 g/L of K2HPO3 is 32 mM. 5 g/L of KH2PO3 is 42 mM. Thizy et al. discloses

compositions of either potassium phosphite or potassium phosphate in the desired concentration range but fails to teach the specific combination of these two ingredients. Reuveni et al. (J. Phytopathology, 1994) disclose that K_2HPO_4 and KH_2PO_4 are each effective against the fungus E. turcicum on corn and that phosphates have exhibited effectiveness against P. sorphi and S. fulginea on maize and cucumbers, respectively, and are effective in enhancing the growth of plants (See entire document.) It is further taught that 100 mM of K_2HPO_4 or KH_2PO_4 was applied to said corn plants (page 339).

Finally, it is disclosed that phosphates are not only fungicides but also fertilizers (page 338). Reuveni et al. (Plant Pathology, 1995) disclose K_2HPO_4 and KH_2PO_4 are each effective against powdery mildew caused by S. fulginea on cucumber and that phosphates have exhibited effectiveness against E. turcicum and P. sorghi on maize and against various diseases in cucumber and are effective in stimulating plant growth (See entire document.). It is also disclosed that 25 mM of K_2HPO_4 or KH_2PO_4 was applied to said cucumber plants (page 31). Reuveni et al. teach that the properties of phosphates and potassium salts thereof make them appropriate for use as foliar fertilizers (page 31). The two Reuveni et al. references teach the effectiveness of potassium phosphate as a fungicide against several types of fungi at a concentration of

25 mM. However, there is no teaching of combining potassium phosphite and potassium phosphate into a single composition at the claimed concentration as an effective fungicide and fertilizer.

Dunstan et al. disclose that the active toxophore of fosetyl-AL is phosphonate produced by catabolism of fosetyl-Al in the plant (page 205). Additionally, it is taught that phosphonate supplied as the potassium salt (Foli-r-fos ®) provides as effective control of many pathogens as fosetyl-Al (page 205). Thus, Dunstan et al. emphasizes the effectiveness of phosphate ion as an antifungal agent but there is no teaching of the combination of potassium phosphite and potassium phosphate.

The cumulative teachings of Thizy et al., Reuveni et al. '94 and '95, and Dunstan disclose the effectiveness of potassium phosphite and potassium phosphates individually as possessing both fungicidal and fertilizer activities but do not teach the specific combination of these two ingredients in the stated concentrations. The Fenn dissertation (1986) provides the missing piece of the obviousness rejection by disclosing the combination of phosphite and phosphate ion in a single composition for the treatment of fungal infections. The Fenn dissertation further teaches that fosetyl-Al and phosphorus acid (H3PO3) (from which phosphate ions are derived) are effective fungicides in the presence of potassium phosphates (pages 63 - 72). It is further disclosed that the potential antagonistic effects of tissue phosphate levels on in vivo efficacy of phosphonate compounds have probably been over emphasized (page 72).

Additionally, it is taught in Fenn that in some cases disease control with H3PO3 in tomato seedlings inoculated with P. palmivora was enhanced when higher concentrations of phosphate were included in the treatments and that at some concentrations of H3PO3, inhibition of mycelial growth in vitro was greater at 45 mM than at 15 mM phosphate with P. capsici and P. parasitica var. nicotinanae (Ibid, pages 72 - 73).

Walker et all. also discloses that a combination of potassium dihydrogen orthophosphate (phosphate) and Foli-R-Fos 200 AC® (potassium phosphite), inhibited in vitro the growth of fungi and that the growth of Phytophthora cactorum, P. cinnamomL P. citrophthora, P. megasperma and Pythium ultimum, and was subject to significant PO3/PO4 interactions.

The Fluid Fertilizer Manual discloses that higher crop yields can be maintained by application of mixtures of pesticides, such as fungicides, and fluid fertilizers which

control fungi while fertilizing the crop (Ch. 6, Section I, page 6-1). It is disclosed that one of the advantages of fluid fertilizers is the ease of preparation of stable, uniform mixes of fluid fertilizer with chemicals than can be applied uniformly and that this advantage greatly facilitates the simultaneous application of fertilizer and pesticide (Ch. 6, Section 1, 1.2, page 6-2). It is taught that it is known to combine fluid fertilizer with fungicides (Ibid.) It is disclosed that the adoption of applying pesticides with fluid fertilizer is attributed to the savings of time, money and other resources, which is sound

agronomic management (Ibid.) It is taught that the combination of pesticide with fluid fertilizer has been shown to enhance crop growth synergistically because the presence of fertilizer makes possible quick, vigorous growth of the crop enabling the crop to more effectively compete with pests held in check by the pesticide, and/or the presence of the pesticides increases effective utilization of fertilizer (Ibid.) Finally, the Fluid Fertilizer Manual teaches that in some cases, applying fertilizer with pesticides will enhance the effectiveness of the pesticide on its target (Ibid.)

In summary, Thizy et al.; Reuveni et al. ('94 and '95), Dunstan et al. collectively teach that potassium phosphates and potassium phophites are effective individually as fungicides against Ascomycetes fungi and as fertilizers in concentrations that fall within the claimed amounts. Additionally, Fenn and Walker each disclose a composition of both phosphite and phosphate ions which were used as antifungal agents. Fenn further discloses that in some cases fungal disease control with phosphite can be enhanced with 45 mM phosphate. Walker discloses a fungicidal composition containing at most

10 mM potassium phosphite and 10 mM potassium phosphate. The Fluid Fertilizer Manual teaches that there is sound basis generally for combining fertilizers with pesticides which includes fungicides.

The difference between the above prior art and the claimed invention is that the prior art does not expressly disclose a method of controlling fungus disease in plants in vivo by applying to the plants the claimed amount (about 20 mM to 5% vol/vol) of

potassium phosphonate salts in combination with potassium phosphate salts. However, the prior art amply suggests the same combination of the instant invention since the prior art discloses that the combination of potassium phosphonate and potassium phosphite salts exhibit in vitro activity against fungi (Fenn and Walker). The prior art further discloses that potassium phosphonates salts and potassium phosphites salts are each effective fungicides and fertilizers (Thizy et al., Reuveni et al. '94 and '95; Dunstan et al.). The combination of fertilizers and fungicides as a single product for application on to plants is an efficient agronomic strategy (Fluid Fertilizer Manual). As such it would have been well within the skill of one of ordinary skill in the art to have applied the combination of potassium phosphonate salts and potassium phosphate salts to plants with the expectation that said combination would have been effective in promoting the growth of plants and controlling fungal infections. Further, one of ordinary skill in the art would have been motivated to vary the amount or concentration of said salts, including the amounts or concentrations falling within the claimed values, as desired, depending on the susceptibility of the fungus, the extent of disease, the host plant and the climate conditions. This would have simply been routine experimentation by the artisan to find the optimal concentration of phosphite and phosphate.

Therefore, the claimed invention, as a whole, would have been prima facie obvious to one of ordinary skill in the art at the time of the invention was made, because every element of the invention has been collectively taught by the combined teachings of the references and clear motivation existed to combine the references.

Claims 1,2 rejected under 35 U.S.C. 103(a) as being unpatentable over Thyzy et al. (4,075,324.

Thyzy et al.-4,075,324; Reuveni et al. '94 ; Reuveni et al. '95 ; Dunstan et al.; Fenn Dissertation ; and Walker as set forth above, and further in view of Lovatt - 5,514,200.

As explained above, the combined references of Thizy et al.; Reuveni et al. '94 Reuveni et al. '95 ; Dunstan et al ; Fenn Dissertation ; Walker; and The Fluid Fertilizer Manual render obvious fungicidal compositions comprising potassium phosphite and potassium phosphate at a concentration of about 20 mM to 5% vol/vol, or at least about 20 mM and a ratio of phosphite to phosphate ion of 0.001 to 1,000. The only substantial difference between the claimed compounds and instant claims is the inclusion of a pH limitation: "having a pH equal to or less than 7.0" or "having a pH of less than 7.0." The above references are primarily silent with regard to the desired pH of such solutions. However, Lovatt (5,514,200) discloses that a "foliage-acceptable pH for phosphorus

uptake usually ranges between about 5.0 to about 7.0 ..." Lovatt explains that "At higher pH, between about 7.0 to about 7.5 there is reduced uptake of nutrients, although generally there is no plant damage." See column 3, lines 26 - 33 and column 4, lines 19 - 32. Therefore, the person of skill in the art with the above references before him, would have found it obvious to have adjusted the pH of the fungicidal composition of potassium phosphite and potassium phosphate to either "less that 7.0" or to "equal to

or less than 7.0" in order to prevent any damage to the plant and to prevent any reduction in the uptake of phosphorus by the plant. For the same reasoning, it would have also been obvious to have applied these same compositions with an approxi\-mately neutral pH to plants in order to control fungal disease and promote plant growth at the same time. The artisan would have had a reasonable expectation of success in controlling fungal disease and in promoting plant growth because the prior art teaches that both potassium phosphite and potassium phosphate are recognized fertilizers and fungicides.

Double Patenting

Claims 1,3-14 STAND rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-12 of U.S. Patent No. US006509041B2 . Although the conflicting claims are not identical, they are not patentably distinct from each other because the patent claims encompasses the instant claims & would anticipate them.

Claims 1, 3-5,9-11 STAND rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim2,4,5 of U.S. Patent No. 5800837. Although the conflicting claims are not identical, they are not patentably distinct from each other because the patent claims encompass the instant

compositions, ratios, & concentrations, thus besides stimulating growth, the patent would inherently meet the instant claim to control fungus.

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NEIL LEVY whose telephone number is 571-272-0619. The examiner can normally be reached on Tuesday-Friday, 7 AM to 5:30 PM EST..

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, ROBERT A. WAX can be reached on 571-272-0623. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/NEIL LEVY/ 4/23/2010
Primary Examiner, Art Unit 1615

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Chromosomal Aberrations Associated with Mutations to Bacteriophage Resistance in *Escherichia coli*

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ABSTRACT

CURTISS, ROY, III (University of Chicago, Chicago, Ill., and Oak Ridge National Laboratory, Oak Ridge, Tenn.). Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28–40. 1965.—Ten types of mutants of *Escherichia coli* K-12 resistant to bacteriophage T_3 have been isolated, and several of these types have been studied genetically. Many of the */3,4,7*, */3,4,7*,λ, and */3,4,7*,λ,$pro^-_{1,2}$ mutants were unstable, changing to complete sensitivity to T_4. The results with strains having */3,4,7*,λ,$pro^-_{1,2}$ mutations were compatible with the hypothesis that this mutation caused a single break in the circular chromosome which prevented the normal association in the inheritance of the outside markers leu^+ and lac^+. When sensitivity to T_4 was regained, association in the inheritance of outside markers was restored, and the resulting */3,7*,λ,$pro^-_{1,2}$ mutation behaved genetically as a deletion. The */3,7*,λ,$pro^-_{1,2}$ and */3,4,7*,λ,$pro^-_{1,2}$ mutations caused positive interference, inhibition of genetic recombination in regions adjacent to them, and the formation of unstable partial diploid recombinants. One group of */3,4,7*,λ mutations did not occur in the *leu* to *try* region of the bacterial genome. Other */3,4,7*,λ mutations in F^- bacteria prevented the joint inheritance of the outside markers lac^+ and gal^+, presumably by breakage of the circular chromosome. Hfr and F^+ strains with */3,4,7*,λ mutations at this locus were unable to conjugate; therefore, a complete genetic analysis of the effects of this */3,4,7*,λ mutation could not be done.

Because *Escherichia coli* K-12 is sensitive to all the T phages and can be used to study gene transfer by conjugation (Lederberg, 1947), it was possible to study the genetic properties of phage-resistant mutants in this strain of *E. coli*. The following linkage relationships have been established for phage-resistance mutations: *leu–/1,5–lac–/6* (Lederberg, 1947); *str*^{r}*–/*λv*,mal*$_1^-$ (Lederberg, 1955); *gal–ura–cysB–anthranilic acid–/1,try*$^-$ (Weinberg, 1960; Curtiss, *unpublished data*); and *str*^{r}*–/3–/*λv*,mal*$_1^-$ (Hayes, 1957; Curtiss, 1962).

In this communication, further work is reported on phage-resistant mutants in *E. coli* K-12. A genetic analysis of */3,4,7,*λ*pro*$^-$ and */3,4,7,*λ mutations has been made which indicates that these mutations are chromosomal aberrations. Postzygotic elimination (Nelson and Lederberg, 1954), positive interference, and inhibition of recombination events have been associated with these aberrations.

[1] Present address: Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

MATERIALS AND METHODS

Media. The following media were used: medium ML, containing NH_4Cl, 0.5%; NH_4NO_3, 0.1%; Na_2SO_4, 0.2%; K_2HPO_4, 0.9%; KH_2PO_4, 0.3%; and $MgSO_4 \cdot 7H_2O$, 0.01%; and medium MA, prepared by adding 2× ML to an equal volume of 3.0% melted agar. A carbon source at 0.5% final concentration and desired growth factor supplements were added to ML and MA. The amino acid and vitamin supplements were purchased commercially and were added at optimal concentrations. Streptomycin sulfate was used at a final concentration of 200 μg/ml. Buffered saline contained: NaCl, 0.85%; KH_2PO_4, 0.03%; and Na_2HPO_4, 0.06% (when used as a diluent, gelatin was added to a concentration of 100 μg/ml). EMB agar contained Tryptone (Difco), 0.8%; yeast extract, 0.1%; NaCl, 0.5%; eosin Y, 0.04%; methylene blue, 0.0065%; and agar, 1.3%. Just prior to pouring plates, the desired sugar was added to give a 1.0% final concentration, and K_2HPO_4 was added to give a 0.2% final concentration. Penassay agar, Penassay broth, and L broth (Lennox, 1955) were employed as complete media.

Bacteria. Table 1 lists the *E. coli* K-12 strains used. Bacteria were maintained on Penassay

TABLE 1. *Escherichia coli K-12 strains*

Strain no.	Mating type	Genetic markers[a]														Derivation
		thr	*leu*	T_1	pro_1	pro_2	*lac*	pro_3	T_6	ade_2	*gal*	*try*	*str*	*met*	*thi*	
χ12	F^-	−	−	s	+	+	−2	+	s	+	−2	+	s	+	−	W945
χ57	Hfr H	+	+	s	+	+	+	+	s	+	+	+	s	+	−	3000
χ80	F^+	+	+	s	−[c]	−[c]	+	+	s	+	+	+	s	−	+	χ11
χ101	F^+	+	+	s	+	+	+	+	s	+	+	+	s	+	−	χ12
χ114	F^-	−	−	s	+	+	−2	+	r	+	−2	−	r	+	−	χ12
χ137	F^-	−	−	r	−[d]	−[d]	−y	+	r	+	+	+	r	+	−	C600
χ148	F^-	+	−	r	+	+	−2	+	r	−	−2	−	r	+	−	χ114
χ188	F^-	+	−	r	−3	+	−2	+	r	−	−2	−	r	+	−	χ148
χ212	F^-	+	−	r	+	+	−g	−2	r	−	+	−	r	+	−	χ148
χ278	F^-	−	−	r	+	−9	−y	+	r	+	+	+	r	+	−	C600

[a] The genetic markers are arranged in the order in which they occur on the chromosome. The following abbreviations are used: *thr*, threonine; *leu*, leucine; *pro*, proline; *lac*, lactose; *ade*, adenine; *gal*, galactose; *try*, tryptophan; *str*, streptomycin; *met*, methionine; *thi*, thiamine; +, ability to synthesize or utilize; −, inability to synthesize or utilize; s, sensitive; and r or /, resistant. Numbers and letters for pro_1^-, pro_2^-, lac^-, pro_3^-, and gal^- mutations are isolation designations. The mutation T_1^r confers resistance to both T_1 and T_5. All strains were nonlysogenic for λ.

[b] W945 (Cavalli-Sforza and Jinks, 1956) was received from M. L. Morse; 3000 (Pardee, Jacob, and Monod, 1959), from N. M. Schwartz; and C600 (Appleyard, 1954), from J. J. Weigle. The omitted intervening steps in each derivation involved penicillin enrichment for ultraviolet-induced auxotrophic mutations, spontaneous selection of mutations to phage and streptomycin resistance, spontaneous reversions to prototrophy or ability to ferment, or a combination of these.

[c] Strain χ80 is */3,4,7,λ,*$pro_{1,2}^-$ (ϕ,$4^r pro_{1,2}^-$).

[d] Strain χ137 is */3,7,λ,*$pro_{1,2}^-$ ($\phi^r pro_{1,2}^-$).

agar slants at 4 C, and were transferred at 2-month intervals.

Bacteriophages. The seven T phages (Demerec and Fano, 1945) were grown on *E. coli* B. The growth of T_3 and T_7 on *E. coli* B was essential, since host-range mutants for */3* and */7* mutants of *E. coli* K-12 made up 1 to 10% of the phage population in lysates repeatedly grown on *E. coli* K-12. Phages λ^{++} (Kaiser, 1955) and λv (Lederberg and Lederberg, 1953) were grown on C600. P1*kc* (Lennox, 1955) and R17 (Paranchych and Graham, 1962) were grown on χ57 or χ101. All phage lysates were stored over chloroform at 4 C. The techniques used for phage experiments were described in the references cited above and by Adams (1959).

Isolation of phage-resistant mutants. Phage-resistant mutants were isolated on Penassay agar containing 0.8% NaCl by the spread-plate technique described by Demerec and Fano (1945). Mutants were purified from phage by three serial single-colony isolations or by growing in the presence of diluted antiphage serum, or by both methods.

Mating procedure. Bacteria were grown to log phase either in L broth or in appropriately supplemented ML at 37 C with aeration. ML-grown cultures were sedimented and resuspended in a minimal mating medium (pH 6.3) as described by Fisher (1957). Matings were performed at 37 C in a stationary 250-ml Erlenmeyer flask containing a total volume of 10 ml. F^- bacteria were at a titer of 3×10^8 to 4×10^8 per milliliter, and were always at a 10-fold or greater excess in crosses with Hfr donors.

Interruption of mating was accomplished by a modification of the procedure described by Hayes (1957). Samples from the mating mixture were diluted into ultraviolet-irradiated T_6, and then unadsorbed T_6 was neutralized with T_6 antiserum. Violent agitation with a Vortex Junior mixer was sometimes used prior to T_6 treatment.

Recombination percentages were calculated by dividing the recombinant titer × 100 by the titer of the donor strain in the mating mixture at the beginning of the experiment. Recombinant titers in crosses with phage-resistant mutants were based on the percentage of prototroph colonies which contained haploid recombinants exclusively (see discussion of Fig. 1). After purification of recombinants, unselected nutritional and fermentation characters were scored by replica plating (Lederberg and Lederberg, 1952). Phage resistance was scored by streaking recombinant cultures against phage on EMB containing 0.1% glucose (Zinder, 1958), since even small amounts of lysis could be detected by the red discoloration at the juncture of the phage and bacterial streaks. These tests for phage resistance were always read after 6 to 8 hr of incubation at 37 C, because many of the phage-resistant mutants were mucoid and slime production made accurate scoring impossible after overnight incubation.

TABLE 2. *Mutants of Escherichia coli K-12 resistant to bacteriophage T_3*

Type of resistance	No. isolated*
/3	65
/3,λ	5
/3,4	25
/3,4,λ	3
/3,7	11
/3,7,λ (ϕ^r)†	9
/3,4,7	54
/3,4,7,λ (ϕ,4^r)†	57
/3,7,λ,pro^- ($\phi^r pro^-$)†	4
/3,4,7,λ,pro^- (ϕ,4$^r pro^-$)†	11
Total	244

* The number isolated cannot be equated to mutant frequency.

† The symbol ϕ^r will be used in the text as a shorthand notation for joint resistance to T_3, T_7, and λ. The symbol ϕ^s indicates sensitivity to T_3, T_7, and λ.

RESULTS

Types of phage-resistant mutants. Table 2 lists the types of mutants obtained by selection with T_3 in *E. coli* K-12. By contrast, in *E. coli* B resistance to T_3 and T_4 are inseparable, so that, if resistance to λ is ignored, the types */3*, */3,7*, and */3,7,pro⁻* do not occur. All of the */3* and */3*,λ mutants had a rough colony type identical to the parental sensitive strain. The mutants in the other classes gave a continuum of colony morphologies from rough to very mucoid. In general, the types showing resistance to T_4 were more mucoid than those not having resistance to T_4.

In fluctuation tests (Luria and Delbrück, 1943) on the origin of mutants resistant to T_3, large fluctuations were observed, indicating that the mutants were of spontaneous origin.

Those T_3-resistant mutants having resistance to T_4 sometimes lost it after several transfers on slants or during selection for other mutations. The */3,4,7*, */3,4,7*,λ, and */3,4,7*,λ,pro^- mutants frequently changed to */3,7*, */3,7*,λ, and */3,7*,λ,pro^-, respectively. Most of the phage-resistant prolineless mutants were originally isolated as */3,4,7*,λ,pro^- and, although they were usually stable during repeated transfers, they invariably changed to */3,7*,λ,pro^- when any other forward mutation was isolated, whether it was a mutation to drug, phage, or analogue resistance or a mutation to auxotrophy. Very infrequently, restoration of T_4 sensitivity accompanied revision of auxotrophic mutations to prototrophy. No proline-independent revertants were detected in reversion studies with 11 */3,7*,λ,pro^- and */3,4,7*,λ,pro^- mutants.

All of the phage-resistant mutants used in the experiments reported in the following sections were resistant to T_3, T_7, and λ. To simplify the presentation of these results, the symbol ϕ^r will be used to indicate joint resistance to T_3, T_7, and λ (see Table 2).

Time of entry for Hfr H genetic markers. For a comparison with data presented below, the linkage relationships of the genetic markers employed in this research are presented in Table 3. Hfr donor bacteria transfer their chromosome to F^- recipient bacteria in an oriented sequential order. The time when a given Hfr marker is first transferred to F^- cells is the time of entry for that marker. The data in Table 3 are for the Hayes Hfr strain. The Cavalli Hfr strain, which transfers its chromosome in the order *ade₃* T_6 *lac leu*..., has also been used. The distances between markers were essentially the same as found for Hfr H.

E. coli K-12 has three *pro* loci, called pro_1, pro_2, and pro_3. The *lac*-pro_3 region can be cotransduced (Schwartz, 1963; Markovitz, 1964;

TABLE 3. *Time of entry for Hfr H genetic markers**

Marker transferred from Hfr H	Time of entry†	Distance between markers
		min
leu^+	8.7 (13)	
		2.4
T_1^s	11.1 (3)	
		4.0
pro_1^+	15.1 (3)	
		2.1
pro_2^+	17.2 (2)	
		0.6‡
$\phi^s pro_{1,2}^+$	17.5 (2)	
		0.3
lac^+	17.8 (10)	
		0.2
pro_3^+	18.0 (6)	
		2.0
T_6^s	20.0 (2)	
		2.5
ade_3^+	22.5 (6)	
		2.3
gal^+	24.8 (3)	
		9.7
try^+	34.5 (2)	

* Matings interrupted with ultraviolet-irradiated T_6. F^- strains used were χ114, χ137, χ148, χ188, χ212, and χ278.

† Numbers in parentheses refer to number of determinations.

‡ Distance between pro_2^+ and lac^+.

TABLE 4. *Inheritance of $\phi,4^s$ marker from Hfr H*[a]

Control cross		Experimental cross	
Hfr H *thr⁺ leu⁺ lac⁺ gal⁺ try⁺ str*^{s} χ114 *thr⁻ leu⁻ lac⁻ gal⁻ try⁻ str*^{r}		Hfr H *thr⁺ leu⁺ lac⁺* $\phi,4^s$ *gal⁺ try⁺ str*^{s} χ114 $\phi,4^r$ *thr⁻ leu⁻ lac⁻* $\phi,4^r$ *gal⁻ try⁻ str*^{r}	
Recombinant class selected[b]	Relative recombinant frequency[c]	Recombinant class selected[b]	Relative recombinant frequency[d]
***thr⁺ leu⁺ lac⁺ str**^{r}*	100	***thr⁺ leu⁺ lac⁺*** $\phi,4^r$ ***str**^{r}*	100
***gal⁺ try⁺ str**^{r}*	22.7	$\phi,4^r$ ***gal⁺ try⁺ str**^{r}*	23.1
***thr⁺ leu⁺ lac⁺ gal⁺ try⁺ str**^{r}*	10.0	***thr⁺ leu⁺ lac⁺*** $\phi,4^s$ ***gal⁺ try⁺ str**^{r}*	0.083

[a] The bacteria were grown in minimal media plus succinate and mated in appropriately supplemented minimal mating media with succinate for 60 min. The *thr⁺* marker is closely linked to *leu⁺* and enters 0.5 min before *leu⁺* in crosses with Hfr H.

[b] The markers employed for selection of recombinants are indicated in bold-face type. The recombinants were picked, purified, and then scored for unselected markers.

[c] Actual percentage of *thr⁺ leu⁺ lac⁺ str*^{r} recombinants was 4.1%.

[d] Actual percentage of *thr⁺ leu⁺ lac⁺* $\phi,4^r$ *str*^{r} recombinants was 3.9%.

Curtiss and Charamella, *unpublished data*), as can the *pro₂-lac* region (Curtiss and Charamella, *unpublished data*). The pro_1 to *lac* region cannot be cotransduced with P1*kc*. All pro_3^- mutants crossfeed pro_1^- and pro_2^- mutants (Pittard, *unpublished data;* Curtiss and Charamella, *unpublished data*), and there is no crossfeeding between pro_1^- and pro_2^- mutants (Curtiss and Charamella, *unpublished data*). Hfr P4X6 transfers pro_1^+ as the first genetic marker and pro_2^+ as the last genetic marker, whereas Hfr OR1 (Curtiss, 1964*a*) transfers pro_2^+ first and pro_3^+ last (Curtiss and Charamella, *unpublished data*). Time of entry experiments with Hfr H (Table 3), Hfr Cavalli, and Hfr OR1 (Curtiss, 1964*a*), and subsequent recombinant analyses, showed that the *lac* locus was between the pro_2 and pro_3 loci.

No *pro⁺* recombinants were obtained in crosses between $\phi^r pro^-$ or $\phi,4^r pro^-$ mutants and pro_1^- or pro_2^- mutants, whereas *pro⁺* recombinants were obtained in crosses with pro_3^- mutants. All pro_3^- mutants cross-fed all $\phi^r pro^-$ and $\phi,4^r pro^-$ mutants. The time of entry for both the pro_1^+ and the $\phi^s pro^+$ markers from Hfr Cavalli was about 12 to 13 min. It is, therefore, concluded that the phage-resistant, prolineless mutants have a deletion of 2 to 2.5 min of the bacterial chromosome which includes the pro_1 and pro_2 loci. All pro_1^-, pro_2^-, $\phi^r pro_{1,2}^-$, and $\phi,4^r pro_{1,2}^-$ mutations were complemented by the exogenote from a partial diploid strain in which the exogenote complemented the *leu* to pro_2 region of the chromosome, but not the *lac* or pro_3 region (Curtiss, 1964*b*). A test for cotransducibility of the pro_1 and pro_2 loci by employing $\phi^r pro_{1,2}^-$ recipients could not be done, since all $\phi^r pro_{1,2}^-$ mutants were resistant to P1*kc*.

Genetic recombination with F⁻ strains having $\phi,4^r$ *or* $\phi,4^r pro_{1,2}^-$ *mutations.* Experiments designed to map the $\phi,4^r$ mutation with respect to other markers gave anomalous results. In *E. coli* K-12 the *lac* and *gal* loci are about 7 min apart (Table 3), and in crosses with Hfr H the *lac⁺* and *gal⁺* markers are frequently inherited jointly. In the experimental cross between Hfr H and a $\phi,4^r$ mutant obtained from χ114 (Table 4), the relative frequency of *thr⁺ leu⁺ lac⁺ gal⁺ try⁺ str*^{r} recombinants (0.083) was less than 1% of the relative frequency of this same recombinant class in the control cross between Hfr H and χ114 (10.0). In contrast, the relative frequencies of *gal⁺ try⁺ str*^{r} recombinants were the same, regardless of whether the $\phi,4^r$ mutation was present or not (Table 4). In the cross with the χ114 $\phi,4^r$ mutant, only those recombinants which had inherited both the *lac⁺* and *gal⁺* markers from Hfr H also inherited the Hfr H $\phi,4^s$ allele. In this cross, all of the *thr⁺ leu⁺ lac⁺ str*^{r} and *gal⁺ try⁺ str*^{r} recombinants tested had inherited the $\phi,4^r$ marker from the F⁻ parent. Results similar to those in Table 4 were obtained in several other crosses with the χ114 $\phi,4^r$ mutant. All of these results indicated that the $\phi,4^r$ mutation in χ114 was linked between the *lac* and *gal* loci and somehow interfered with the joint inheritance of the *lac⁺* and *gal⁺* alleles from Hfr H.

Time of entry experiments with the χ114 $\phi,4^r$ mutant showed that the *thr⁺ leu⁺*, *lac⁺*, *gal⁺*, and *try⁺* markers from Hfr H were transferred in the order and at the times expected for Hfr H (see Table 3). It was impossible to determine a

TABLE 5. *Inheritance of* $\phi,4^s pro^+_{1,2}$ *marker from Hfr H*[a]

Control cross		Experimental cross	
Hfr H *leu*$^+$ *lac*$^+$ *ade*$_3^+$ *str*^{s} χ148 *leu*$^-$ *lac*$^-$ *ade*$_3^-$ *str*^{r}		Hfr H *leu*$^+$ $\phi,4^s pro^+_{1,2}$ *lac*$^+$ *ade*$_3^+$ *str*^{s} χ148 $\phi,4^r pro^-_{1,2}$ *leu*$^-$ $\phi,4^r pro^-_{1,2}$ *lac*$^-$ *ade*$_3^-$ *str*^{r}	
Recombinant class selected	Relative recombinant frequency[b]	Recombinant class selected[c]	Relative recombinant frequency[d]
***leu*$^+$ *str*^{r}**	100	***leu*$^+$** $\phi,4^r pro^-_{1,2}$ ***str*^{r}**	100
***lac*$^+$ *ade*$_3^+$ *str*^{r}**	45.1	$\phi,4^r pro^-_{1,2}$ ***lac*$^+$ *ade*$_3^+$ *str*^{r}**	42.6
***leu*$^+$ *lac*$^+$ *str*^{r}**	54.9	***leu*$^+$** $\phi,4^s pro^+_{1,2}$ ***lac*$^+$ *str*^{r}**	1.67

[a] Procedure as for Table 4, except mating was for 90 min.

[b] Actual percentage of *leu*$^+$ *str*^{r} recombinants was 11.3%.

[c] The markers employed for selection of recombinants are indicated in bold-face type. The recombinants were picked, purified, and then scored for unselected markers. Of 100 *leu*$^+$ *str*^{r} recombinants scored, 3 were $\phi,4^s pro^+_{1,2}$ in genotype. Of 100 *lac*$^+$ *ade*$_3^+$ *str*^{r} recombinants scored, 4 were $\phi,4^s pro^+_{1,2}$ in genotype (3 of these were *leu*$^-$ and 1 *leu*$^+$). Of 100 *leu*$^+$ *lac*$^+$ *str*^{r} recombinants scored, 4 were $\phi,4^r pro^-_{1,2}$ in genotype.

[d] Actual percentage of *leu*$^+$ $\phi,4^r pro^-_{1,2}$ *str*^{r} recombinants was 5.4%.

time of entry for the $\phi,4^s$ marker from Hfr H, since all of the *thr*$^+$ *leu*$^+$ and *lac*$^+$ recombinants remained resistant to T_3, T_4, T_7, and λ regardless of the time when the mating was interrupted. Control experiments detected no lethal zygotic events which might have accounted for the absence of linkage between the *lac* and *gal* loci in the Hfr H $\times$ χ114 $\phi,4^r$ cross. Reversion at the *gal* locus was the same in the presence or absence of the $\phi,4^r$ mutation. Reversion at the *lac* locus was decreased, but still detectable, when a $\phi,4^r$ mutation was present (Curtiss, 1962).

Seven independently isolated F$^-$ $\phi,4^r$ mutants have been analyzed genetically in crosses with Hfr H, and three have demonstrated reduction in the joint inheritance of the Hfr H *lac*$^+$ and *gal*$^+$ alleles. In one of these crosses with a well-marked F$^-$ strain, the $\phi,4^r$ mutation caused a sharp reduction in the joint inheritance of the closely linked *ade*$_3^+$ and *gal*$^+$ Hfr H markers, and most *ade*$_3^+$ *gal*$^+$ recombinants were $\phi,4^s$. This indicated that the $\phi,4^r$ mutation in this strain was between the *ade*$_3$ and *gal* loci. In the other four F$^-$ strains, the $\phi,4^r$ mutations were unlinked to the *leu* to *try* region. The results with $\phi,4^r$ mutations linked between the *lac* and *gal* loci indicated that these mutations were associated with chromosome aberrations. This aberration could be a transposition, an inversion, or a single break in the circular chromosome making it linear. In each case, one of the breaks associated with the aberration would have to be between the *lac* and *gal* loci.

A cross between Hfr H and a $\phi,4^r pro^-_{1,2}$ mutant of χ148 demonstrated that the $\phi,4^r pro^-_{1,2}$ mutation disrupted the normal association in the inheritance of the Hfr H *leu*$^+$ and *lac*$^+$ markers (Table 5). Since only those recombinants which had inherited both *leu*$^+$ and *lac*$^+$ had also received the Hfr H $\phi,4^s pro^+_{1,2}$ allele, it can be concluded that the $\phi,4^r pro^-_{1,2}$ locus is between the *leu* and *lac* loci. Similar results were obtained with two other independently isolated F$^-$ $\phi,4^r pro^-_{1,2}$ mutants. In each case, the results indicated that the $\phi,4^r pro^-_{1,2}$ mutations were associated with some type of chromosomal aberration between the *leu* and *lac* loci.

Genetic recombination with an F$^-$ strain having a $\phi^r pro^-_{1,2}$ *mutation.* Before analyzing the data on genetic recombination from a cross between Hfr H and a F$^-$ $\phi^r pro^-_{1,2}$ mutant, it will be helpful to discuss the results of a control cross, presented in Table 6. In this cross between Hfr H and χ278, *pro*$_2^+$ *str*^{r} recombinants were selected, purified, and then tested for recombination on either side of the *pro*$_2$ locus by scoring for four unselected markers.

In Table 6A, the frequency of recombination per region is calculated. By comparing the per cent recombination per region in the *leu* to T_6 segment with the per cent distance per region in the same interval, it is evident that the amount of recombination in any region was approximately proportional to the length of that region, with the exception of region 4. In another control cross between Hfr H and χ188, in which *pro*$_1^+$ *str*^{r} recombinants were selected, the amount of recombination in any region was directly proportional to the length of that region as determined by Hfr H time of entry experiments. In this cross, there was 16% recombination between *leu* and T_1 for a distance of 17.5%; 32% recombination between T_1 and *pro*$_1$ for a distance of 38.5%; 13% recombination between *pro*$_1$ and *lac* for a

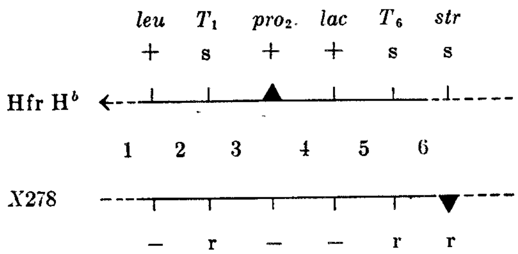
distance of 11%; 19% recombination between *lac* and T_6 for a distance of 15%; and 20% recombination between T_6 and ade_3 for a distance of 18%.

In Table 6B, the double recombinants were analyzed by the method of Maccacaro and Hayes (1961) to determine the type of interference present. Two recombination events are required to incorporate any Hfr H marker into recombinants in *E. coli* K-12. The data analyzed by Maccacaro and Hayes (1961) indicated that, once the first recombination event occurred, the second recombination event occurred more often in regions proximal than in regions distal to the region in which the first recombination event occurred. The analysis in Table 6B indicates that when recombination occurred in region 4, the ratio of recombination in region 3 to that in regions 1 + 2 + 3 was 0.89. When recombination occurred in region 6, the ratio of recombination in region 3 to that in regions 1 + 2 + 3 was only 0.22. Thus, recombination in region 3 was associated more often with recombination in the proximal region 4 than with recombination in the distal region 6. Similarly, the ratio of recombination in region 4 to that in regions 4 + 5 + 6 was 20 times higher when recombination occurred in region 3 (0.60) than when recombination occurred in region 1 (0.03). These results, indicating that there is negative interference in *E. coli* K-12, are in complete accord with those of Maccacaro and Hayes (1961).

Table 7 contains an analysis of $\phi^s pro^+_{1,2}$ str^r recombinants from a cross between Hfr H and χ137. In this cross, there was 70% association in the inheritance of the leu^+ and lac^+ Hfr markers. This contrasts with the almost complete absence of leu^+ lac^+ recombinants in the cross between Hfr H and the $\phi,4^r pro^-_{1,2}$ mutant of χ148 (see Table 5). The parental strain, from which χ137 was derived, was completely resistant to T_4. The change to T_4 sensitivity occurred concomitantly with the selection of a str^r mutation. T_4 had an efficiency of plating of 0.5 on χ137.

The analysis in Table 7A shows that the $\phi^r pro^-_{1,2}$ mutation in χ137 has caused a significant reduction in the frequency of recombination in regions 3, 4, and 5. Based on the lengths of the regions, the amount of recombination in region 3 should be 1.67 times that in region 2. Instead, the amount of recombination in regions 2 and 3 was equal. Based on all the matings with χ137, the amount of recombination in region 4 was about 0.1%. This is 30 times lower than expected. Thus, on either side of the $\phi^r pro^-_{1,2}$ marker in χ137, the frequency of recombination per region was no longer proportional to the length of that region. It should further be noted that, in the control cross with χ278, only 47% of all recombination events occurred in the outside regions 1 and 6 (Table 6A), whereas in the χ137 cross 71% of the recombination events occurred in regions 1 and 6 (Table 7A). Strains χ278 and χ137 were both derived from the same F^- strain (Table 1); therefore, the observed differences can be ascribed to

TABLE 6. *Analysis of pro_2^+ str^r recombinants in a control cross between Hfr H and χ278*[a]



(A) Analysis of recombination by regions[c]

Determination	Recombination events in region						Total
	1	2	3	4	5	6	
Number	210	65	194	127	106	224	926
Per cent	23	7	21	13.5	11.5	24	100
Per cent[d]	—	13	39.5	26	21.5	—	100
Per cent distance[e]	—	21	54	5.5	19.5	—	100

(B) Interference analysis[f]

Region	No. of recombinants with recombination in region 1, 2, or 3, with respect to recombination in region 4, 5, or 6			Total	Ratio of 3 to 1 + 2 + 3
	1	2	3		
4	6	8	112	126	0.89
5	57	16	27	100	0.27
6	139	34	48	221	0.22
Total	202	58	187	447	
Ratio of 4 to 4 + 5 + 6	0.03	0.14	0.60		

[a] Bacteria were grown and mated in L broth. Mating was interrupted after 60 min; 455 $pro_2^+str^r$ recombinants were analyzed.

[b] Distance between markers based on time of entry data for Hfr H (see Table 3).

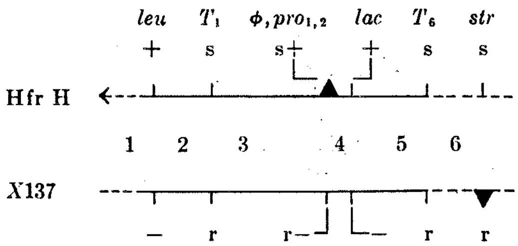
[c] Based on double and quadruple recombination events.

[d] Per cent recombination omitting recombination events in regions 1 and 6.

[e] Per cent distance based on time of entry experiments with Hfr H (Table 3).

[f] Based on double recombination events.

TABLE 7. *Analysis of $\phi^s pro^+_{1,2}$ str^r recombinants from a cross between Hfr H and an F^- with a $\phi^r pro^-_{1,2}$ marker*[a]



(*A*) *Analysis of recombination by regions*[b]

Determination	Recombination events in region						Total
	1	2	3	4	5	6	
Number	247	73	73	0	66	273	732
Per cent	34	10	10	0	9	37	100
Per cent[c]	—	34.5	34.5	0	31	—	100
Per cent distance[d]	—	27	45	3.5	24.5	—	100

(*B*) *Interference analysis*[e]

Region	No. of recombinants with recombination in region 1, 2, or 3, with respect to recombination in region 4, 5, or 6			Total	Ratio of		
	1	2	3		1 to 1 + 2 + 3	2 to 1 + 2 + 3	3 to 1 + 2 + 3
4	0	0	0	0	—	—	—
5	49	6	3	58	0.85	0.10	0.05
6	171	40	43	254	0.67	0.16	0.17
Total	220	46	46	312			
Ratio of 4 + 5 to 4 + 5 + 6	0.22	0.13	0.07				

[a] Bacteria grown and mated in L broth. Mating for 60 min. Distance between markers based on time of entry data for Hfr H (see Table 3) and Hfr Cavalli; 339 $\phi^s pro^+_{1,2}$ str^r recombinants analyzed.

[b] Based on analysis of double and quadruple recombination events. Compare with Table 6A.

[c] Per cent recombination omitting recombination events in regions 1 and 6. Compare with Table 6A.

[d] Per cent distance based on time of entry experiments with Hfr H (Table 3) and Hfr Cavalli. These percentages are different than those in Table 6A since the $\phi^r pro^-_{1,2}$ mutation in χ137 has caused a deletion of the pro_1 to pro_2 segment.

[e] Based on analysis of double recombination events.

differences between the pro_2^- point mutation in χ278 and the $\phi^r pro^-_{1,2}$ deletion mutation in χ137.

The analysis of the $\phi^s pro^+_{1,2}$ str^r recombinants demonstrates that the $\phi^r pro^-_{1,2}$ mutation has caused strong positive interference (Table 7B). Thus, when recombination occurred in the proximal region 5, the ratio of recombination in region 3 to that in regions 1 + 2 + 3 (0.05) was much lower than this same ratio when recombination occurred in the distal region 6 (0.17). Likewise, the ratio of recombination in regions 4 and 5 to that in regions 4 + 5 + 6 was least when recombination occurred in the proximal region 3 (0.07) and greatest when recombination occurred in the distal region 1 (0.22). This positive interference found with χ137 with its $\phi^r pro^-_{1,2}$ mutation sharply contrasts with the negative interference observed with χ278, which has a pro_2^- point mutation (Table 6B).

All the crosses in this section were also done with the Cavalli Hfr with essentially the same results. Thus, the observations cannot be ascribed to the polarity of chromosome transfer by Hfr H, since the Cavalli Hfr transfers the same chromosomal segment in the opposite direction.

To summarize this section, it can be stated that the pleiotropic mutation, $\phi^r pro^-_{1,2}$, causes no disruption in the joint inheritance of outside markers as does the $\phi, 4^r pro^-_{1,2}$ mutation. The $\phi^r pro^-_{1,2}$ mutation behaves like a deletion, since it does not revert and no pro^+ recombinants are obtained in crosses with pro_1^- or pro_2^- donors. This mutation causes positive interference and also interferes with recombination in regions adjacent to it.

Effect of $\phi, 4^r$ and $\phi, 4^r pro^-_{1,2}$ mutations in Hfr and F^+ bacteria. All $\phi, 4^r$ and $\phi, 4^r pro^-_{1,2}$ mutants were independently isolated from various Hfr and F^+ strains and had $\phi, 4^r$ and $\phi, 4^r pro^-_{1,2}$ mutations which were of independent origin from those in F^- strains cited in previous sections. Two-thirds of the F^+ $\phi, 4^r$ mutants isolated failed to yield recombinants in crosses with F^- bacteria (Table 8). A similar result was observed for Hfr $\phi, 4^r$ mutants. Two Hfr $\phi, 4^r$ mutants which failed to yield recombinants were mixed with F^- bacteria and observed by phase-contrast microscopy to detect conjugating pairs (Lederberg, 1956). No conjugating pairs were seen. All of the recently isolated F^+ and Hfr $\phi, 4^r$ mutants which failed to yield recombinants have been tested for sensitivity to the donor specific ribonucleic acid (RNA) phage R17 (Paranchych and Graham, 1962) and found to be resistant. Presumably, in this type of mutant the cell wall has been altered so as to prevent conjugation. Several of the nonconjugating F^+ and Hfr $\phi, 4^r$ mutants

were also mated with F^- $\phi,4^r$ mutants. No recombinants were detected in these matings. Nonconjugating F^+ $\phi,4^r$ mutants were found to act as recipients of genetic material in crosses with phage-sensitive Hfr and F^+ strains.

Those F^+ and Hfr $\phi,4^r$ mutants which yielded normal recombination frequencies were R17-sensitive and had $\phi,4^r$ mutations unlinked to the *leu* to *try* region of the genome (Table 8). All F^+ and Hfr $\phi,4^r pro^-_{1,2}$ mutants tested were fertile (Table 8).

Inheritance of the $\phi,4^r pro^-_{1,2}$ marker from F^+ donors. Table 9 presents data from two crosses employing F^+ $\phi,4^r pro^-_{1,2}$ mutant donors. In cross A, the $\phi,4^r pro^-_{1,2}$ F^+ marker was inherited by 59.5% of the thr^+ leu^+ recombinants. The $\phi,4^r pro^-_{1,2}$ and lac^+ markers from the F^+ donor were associated 6% of the time among those recombinants which had inherited the $\phi,4^r pro^-_{1,2}$ marker (3/47). Normally, the pro_2 and *lac* loci were linked (see Table 6).

Cross B (Table 9) employed a $\phi,4^r pro^-_{1,2}$ mutant isolated from χ101. Strain χ101 was co-isogenic with χ148 (see Table 1), and, therefore, any anomalies in linkage could be ascribed to the $\phi,4^r pro^-_{1,2}$ mutation. Testing of the χ101 $\phi,4^r pro^-_{1,2}$ donor culture revealed that 8% of the cells were sensitive to T_4, and, therefore, $\phi^r pro^-_{1,2}$ in genotype. Among the leu^+ str^r recombinants the $\phi,4^r pro^-_{1,2}$ or $\phi^r pro^-_{1,2}$ marker was inherited 58% of the time. Of the 45 leu^+ str^r recombinants which had inherited the F^+ $\phi,4^r pro^-_{1,2}$ marker, only 5 were lac^+, whereas of the 13 which had inherited the F^+ $\phi^r pro^-_{1,2}$ marker 12 were lac^+. Among the lac^+ str^r recombinants, 17% inherited either the $\phi,4^r pro^-_{1,2}$ or $\phi^r pro^-_{1,2}$ F^+ marker. However, of these 17 lac^+ str^r recombinants 15 were $\phi^r pro^-_{1,2}$ and only 2 were $\phi,4^r pro^-_{1,2}$ in genotype. These results indicated that the F^+ $\phi,4^r pro^-_{1,2}$ mutation interfered with the joint inheritance of the F^+ leu^+ and lac^+ markers, whereas the F^+ $\phi^r pro^-_{1,2}$ mutation did not prevent the normal association in the inheritance of the F^+ leu^+ and lac^+ markers. Since the χ101 $\phi,4^r pro^-_{1,2}$ mutant was completely resistant to T_4 upon initial isolation, it is evident that the change in state from $\phi,4^r pro^-_{1,2}$ to

TABLE 8. *Effect on recombination frequency of $\phi,4^r$ and $\phi,4^r pro^-_{1,2}$ mutations in Hfr and F^+ bacteria**

Mating	No. of donors in category	Recombination frequency as percentage of control†
$F^+ \times F^-$ (control)		
F^+ $\phi,4^r \times F^-$	3	50–200
	3	5–20
	12	<1
F^+ $\phi,4^r pro^-_{1,2} \times F^-$	4	40–300
Hfr $\times F^-$ (control)		
Hfr $\phi,4^r \times F^-$	4	30–180
	18	$<10^{-4}$
Hfr $\phi,4^r pro^-_{1,2} \times F^-$	4	50–240

* All matings were for 60 min in broth. The $\phi,4^r$ and $\phi,4^r pro^-_{1,2}$ mutants were obtained from several different F^+ and Hfr strains. The parent donor strain was used as a control in each case.

† Values of <1% for F^+ crosses and $<10^{-4}$% for Hfr crosses indicate that no recombinants were detected.

TABLE 9. *Inheritance of the $\phi,4^r pro^-_{1,2}$ marker from F^+ donors*[a]

Recombinant type[b]	No.	Per cent
Cross A[c]		
$\boldsymbol{met^+}$ $\boldsymbol{thr^+}$ $\boldsymbol{leu^+}$ $\phi,4^r pro^-_{1,2}$ lac^+	3	3.8
$\boldsymbol{met^+}$ $\boldsymbol{thr^+}$ $\boldsymbol{leu^+}$ $\phi,4^r pro^-_{1,2}$ lac^-	44	55.7
$\boldsymbol{met^+}$ $\boldsymbol{thr^+}$ $\boldsymbol{leu^+}$ $\phi,4^s pro^+_{1,2}$ lac^-	32	40.5
Total	79	100.0
Cross B[d]		
$\boldsymbol{leu^+}$ $\phi,4^r pro^-_{1,2}$ lac^+ $\boldsymbol{str^r}$	5	5
$\boldsymbol{leu^+}$ $\phi^r pro^-_{1,2}$ lac^+ $\boldsymbol{str^r}$	12	12
$\boldsymbol{leu^+}$ $\phi,4^r pro^-_{1,2}$ lac^- $\boldsymbol{str^r}$	40	40
$\boldsymbol{leu^+}$ $\phi^r pro^-_{1,2}$ lac^- $\boldsymbol{str^r}$	1	1
$\boldsymbol{leu^+}$ $\phi,4^s pro^+_{1,2}$ lac^- $\boldsymbol{str^r}$	33	33
$\boldsymbol{leu^+}$ $\phi,4^s pro^+_{1,2}$ lac^+ $\boldsymbol{str^r}$	9	9
Total	100	100
leu^+ $\phi,4^r pro^-_{1,2}$ $\boldsymbol{lac^+}$ $\boldsymbol{str^r}$	1	1
leu^+ $\phi^r pro^-_{1,2}$ $\boldsymbol{lac^+}$ $\boldsymbol{str^r}$	14	14
leu^- $\phi,4^r pro^-_{1,2}$ $\boldsymbol{lac^+}$ $\boldsymbol{str^r}$	1	1
leu^- $\phi^r pro^-_{1,2}$ $\boldsymbol{lac^+}$ $\boldsymbol{str^r}$	1	1
leu^- $\phi,4^s pro^+_{1,2}$ $\boldsymbol{lac^+}$ $\boldsymbol{str^r}$	72	72
leu^+ $\phi,4^s pro^+_{1,2}$ $\boldsymbol{lac^+}$ $\boldsymbol{str^r}$	11	11
Total	100	100

[a] The bacteria were grown and mated in broth. After 60 min for mating, the cells were centrifuged and resuspended in buffered saline before plating on selective medium.

[b] The markers employed for selection of recombinants are indicated in bold-face type. The recombinants were picked, purified, and then scored for unselected markers.

[c] Cross A:
χ80 F^+ met^- thr^+ leu^+ $\phi,4^r pro^-_{1,2}$ lac^+
χ12 F^- met^+ thr^- leu^- $\phi,4^s pro^+_{1,2}$ lac^-

[d] Cross B:
χ101 F^+ leu^+ $\phi,4^r pro^-_{1,2}$ lac^+ str^s
χ148 F^- leu^- $\phi,4^s pro^+_{1,2}$ lac^- str^r

From the χ101 F^+ culture used in the mating, 100 isolates were tested for T_4 sensitivity. Eight were sensitive to T_4 and therefore $\phi^r pro^-_{1,2}$ in genotype. The leu^+ str^r recombinants were twice as frequent as lac^+ str^r recombinants.

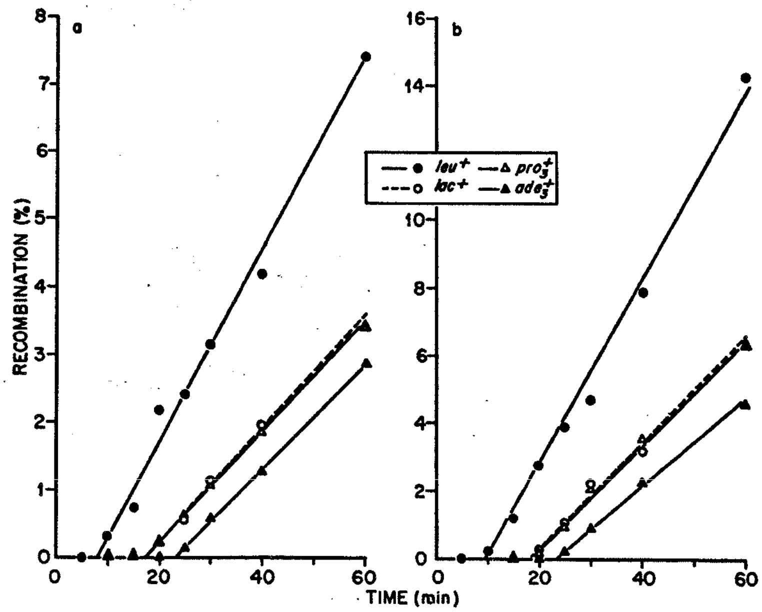


FIG. 1. Time of entry for Hfr (a) and Hfr H $\phi,4^r pro^-_{1.2}$ (b) markers into $\chi 212$ (F^- leu^- lac^- pro_3^- ade_3^- str^r). Bacteria were grown and mated in L broth. Mating was interrupted with ultraviolet-irradiated T_6, and unadsorbed phage was neutralized with antiserum to T_6. The F^- str^r marker was employed in all recombinant selections. Only 10% of the pro^+ recombinants in the Hfr H $\phi,4^r pro^-_{1.2}$ × $\chi 212$ cross were stable.

$\phi^r pro^-_{1.2}$ was responsible for the restoration of the normal association in the inheritance of the outside markers leu^+ and lac^+. The data in Table 9 are in complete accord with the results obtained with an F^- $\phi,4^r pro^-_{1.2}$ mutant (Table 5) and with an F^- $\phi^r pro^-_{1.2}$ mutant (Table 7).

Inheritance of the $\phi,4^r pro^-_{1.2}$ marker from Hfr H. Two independently isolated $\phi,4^r pro^-_{1.2}$ mutants were obtained from Hfr H for use in time of entry experiments. Both mutants were slightly sensitive to T_4 (efficiency of plating of 10^{-3}) and yielded similar results in crosses with various F^- recipients. All nonrecombinant donor isolates and recombinants which had inherited the $\phi,4^r pro^-_{1.2}$ marker demonstrated this same weak sensitivity to T_4.

Figure 1 presents the data for time of entry experiments with Hfr H (a) and with one of the Hfr H $\phi,4^r pro^-_{1.2}$ mutants (b). The time of entry for each marker was the same in both matings while the per cent recombination was twice as high in the Hfr H $\phi,4^r pro^-_{1.2}$ cross.

The results with the Hfr H $\phi,4r^r pro^-_{1.2}$ strain demonstrate another phenomenon observed in all crosses with $\phi^r pro^-_{1.2}$, $\phi,4^r pro^-_{1.2}$, and $\phi,4^r$ mutants. The F^- strain employed in the crosses with Hfr H and the Hfr H $\phi,4^r pro^-_{1.2}$ mutant had a pro_3^- mutation which was closely linked to the $\phi^r pro^-_{1.2}$ locus (see Table 3). Therefore, pro^+ recombinants in the cross with the Hfr H $\phi,4^r pro^-_{1.2}$ mutant should have been infrequent. There are two explanations for the high yield of pro^+ recombinants.

The first explanation is that, after chromosome transfer, the transferred partial chromosome is not immediately integrated to form haploid recombinants but persists as a replicating exogenote. This replicating exogenote could then be lost or integrated at a later cell division. The per cent pro^+ recombinants in Fig. 1b is based on plate counts. The pro^+ colonies varied in size from very small to large. The analysis of pro^+ colonies obtained from platings after 60 min of mating indicated that only 10% contained haploid recombinants exclusively. All of these were large-colony types. Resuspending entire small colonies and then plating on proline-deficient media resulted in the formation of several to 1 million pro^+ colonies per original colony. The sizes of these colonies also showed great variation. Large-colony types, each containing haploid recombinants of one genotype, were sometimes observed among the descendants from one small-colony type. However, different haploid recombinant genotypes were frequently obtained from one original small-colony type. This result is reminiscent of the repeated recombination events observed by Anderson (1958) in his study on cell pedigrees of exconjugants in *E. coli* K-12.

When the small-colony types were resuspended

and plated on proline-deficient media, most of the cells failed to grow. By diluting the colony suspension and plating on Penassay agar, it was shown by replica plating that many of the cells had integrated the $\phi,4^r pro^-_{1,2}$ marker. After several serial small-colony isolations from proline-deficient media to eliminate any contaminating nonrecombinant F⁻ cells, replica plating indicated that nonrecombinant F⁻ cells were still present. Further serial small-colony isolations did not reduce the frequency of these nonrecombinant F⁻ segregants. Therefore, in these instances the transferred exogenote either did not replicate at the same rate as the F⁻ chromosome or was randomly excluded from some descendants at cell division.

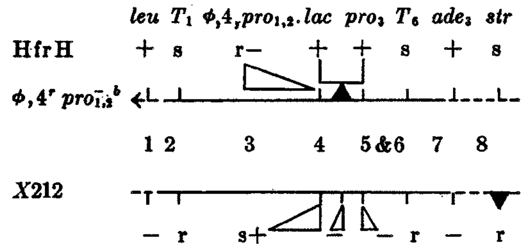
The formation of unstable partial diploids with the eventual loss of the transferred exogenote by integration or exclusion is an example of postzygotic elimination (Nelson and Lederberg, 1954). This phenomenon was observed in all the crosses with $\phi^r pro^-_{1,2}$, $\phi,4^r pro^-_{1,2}$, and $\phi,4^r$ mutants. Therefore, in all crosses with phage-resistant mutants reported in preceding sections, recombinants were purified by picking into buffered saline and then restreaked on the original selective media. The procedure employed eliminated all but haploid recombinants and stable partial diploid strains which were sometimes obtained (Curtiss, 1964*b*). In this manner, it was possible to reisolate all the recombinants picked from the Hfr H × χ212 cross (Fig. 1a). In the Hfr H $\phi,4^r pro^-_{1,2}$ × χ212 mating (Fig. 1b) only 72% of the *leu*⁺, 66% of the *lac*⁺, and 74% of the ade_3^+ colonies could be reisolated upon restreaking.

The second explanation for the high number of *pro*⁺ recombinants in the Hfr $\phi,4^r pro^-_{1,2}$ cross (Fig. 1b) accounts for almost all of the stable pro_3^+ haploid recombinants (10% of the total *pro*⁺ colonies). Of 99 pro_3^+ haploid recombinants analyzed, all were $\phi,4^s pro^+_{1,2}$ and 95 were *lac*⁺. Since the distance between the $\phi,4^r pro_{1,2}$ and *lac* loci is only twice that between the *lac* and pro_3 loci (Table 3), the amount of recombination between the $\phi,4^r pro_{1,2}$ and *lac* loci was much higher than would have been predicted.

The *lac*⁺ *str*ʳ recombinants from the Hfr H $\phi,4^r pro^-_{1,2}$ × χ212 mating (Fig. 1b) are analyzed in Table 10. The summary presented in Table 10A demonstrates that the $\phi,4^r pro^-_{1,2}$ marker has caused a significant reduction in recombination in regions 3, 5, and 6, whereas there was a 10-fold excess in recombination in region 4.

The analysis in Table 10B indicates that there was no negative or positive interference, since a recombination event in one region had no influence on the randomness of the second recombination event. The lack of interference noted in this cross contrasts with the negative interference observed in the control mating between Hfr H and χ278 (Table 6) and the positive interference obtained in the cross with the $\phi^r pro^-_{1,2}$ mutant, χ137 (Table 7). The 10-fold excess in recombina-

TABLE 10. *Analysis of lac⁺ strʳ recombinants from a cross between Hfr H $\phi,4^r pro^-_{1,2}$ and χ212*[a]



(*A*) *Analysis of recombination by regions*[c]

Determination	Recombination events in region 1	2	3	4	5&6	7	8	Total
Number	146	59	43	78	43	111	120	600
Per cent	24	10	7	13	7	19	20	100
Per cent[d]	—	18	13	23	13	33	—	100
Per cent distance[e]	—	21	35	2.5	19.5	22	—	100

(*B*) *Interference analysis*[f]

Region	No. of recombinants with recombination in region 1, 2, 3, or 4, with respect to recombination in region 5 and 6, 7, or 8: 1	2	3	4	Total	Ratio of 1 to 1 + 2 + 3 + 4	2 to 1 + 2 + 3 + 4	3 to 1 + 2 + 3 + 4	4 to 1 + 2 + 3 + 4
5&6	17	5	3	8	33	0.52	0.15	0.09	0.24
7	49	14	8	17	88	0.56	0.16	0.09	0.19
8	51	15	11	27	104	0.49	0.14	0.11	0.26
Total	117	34	22	52	225				

[a] The *lac*⁺ *str*ʳ recombinants analyzed were taken from platings after 60 min of mating (see Fig. 1).

[b] Distance between markers based on time of entry data for Hfr H (see Table 3).

[c] Based on 225 double recombinants, 36 quadruple recombinants, and 1 hextuple recombinant.

[d] Per cent recombination omitting recombination events in regions 1 and 8.

[e] Per cent distance based on time of entry experiments with Hfr H (Table 3) and Hfr Cavalli.

[f] Based on double recombination events.

tion events between the $\phi,4^r pro_{1,2}$ and *lac* loci (region 4) might have prevented the detection of the expected positive interference in this cross.

Discussion

Some $\phi,4^r$ mutations in F^- bacteria caused significant decreases in the joint inheritance of the Hfr H lac^+ and gal^+ markers (Table 4). It was thus suggested that these $\phi,4^r$ mutations were associated with some type of chromosome aberration. This aberration could be a transposition, an inversion, or a single break in the circular chromosome, making it linear. The finding that donor strains with $\phi,4^r$ mutations (presumably linked between the *lac* and *gal* loci) were unable to conjugate (Table 8) made it impossible to clearly differentiate between these alternative hypotheses.

All F^- $\phi,4^r pro^-_{1.2}$ mutations tested caused a decrease in the joint inheritance of the Hfr H leu^+ and lac^+ markers (Table 5). When the $\phi,4^r pro^-_{1.2}$ mutation was in F^+ donors, it also effectively prevented the joint inheritance of the F^+ leu^+ and lac^+ markers (Table 9). However, the spontaneous change from $\phi,4^r pro^-_{1.2}$ to $\phi^r pro^-_{1.2}$ restored linked inheritance of the leu^+ and lac^+ markers (Tables 7 and 9). The simplest way to explain these data with F^+ and F^- $\phi,4^r pro^-_{1.2}$ and $\phi^r pro^-_{1.2}$ mutants is to postulate that the original $\phi,4^r pro^-_{1.2}$ mutation caused a single break in the circular chromosome, making it linear, and that restoration of the circular chromosome was accompanied by a return of T_4 sensitivity. It is difficult to construct a model for restoration of the original linkage if inversions or transpositions are involved. Furthermore, one of the two break points would have to be outside of the *leu* to *try* region, and in one experiment with an F^- $\phi,4^r pro^-_{1.2}$ mutant the inheritance and linkage of markers in other regions of the chromosome was normal.

This model of a single break in the circular chromosome caused by $\phi,4^r pro^-_{1.2}$ mutations readily explains the almost complete absence of leu^+ lac^+ recombinants in the crosses with F^+ $\phi,4^r pro^-_{1.2}$ mutants, since both markers would not be transferred to the same recipient cell. However, when the $\phi,4^r pro^-_{1.2}$ mutation is in the F^- parent it might be expected that the transferred Hfr chromosome, which contains linked leu^+ and lac^+ markers, would restore the circular chromosome and give rise to normal frequencies of leu^+ lac^+ recombinants. Since this is not observed (Table 5), it must be concluded that the F^- *leu* and *lac* loci are physically separated so that the Hfr chromosome segment cannot readily pair with both F^- loci.

This model of a single break in the circular chromosome caused by $\phi,4^r pro^-_{1.2}$ mutations further postulates that the circular chromosome is reformed upon return of T_4 sensitivity. To explain this (fortuitous coincidence?), it must be assumed that the original mutation does not occur in a gene responsible for some structure involved in T_4 infection. Rather, the break in the circular chromosome must interfere with the functioning of a neighboring gene(s) for T_4 sensitivity. Restoration of the circular chromosome could then allow the near-normal expression of this gene(s).

Since the $\phi^r pro^-_{1.2}$ mutation is a deletion (genetically), it is apparent that the original break in the chromosome resulted in a loss of genetic material. Therefore, it is possible that deletion mutations in bacteria arise by a process involving chromosome breakage, loss of genetic material and then rejoining of the free ends.

The data obtained from crosses with F^+ $\phi,4^r pro^-_{1.2}$ mutants and with F^- $\phi,4^r$, $\phi,4^r pro^-_{1.2}$, and $\phi^r pro^-_{1.2}$ mutants are all compatible with the above model. It was reasoned, however, that proof of this theory would require time of entry experiments with Hfr $\phi,4^r pro^-_{1.2}$ mutants. It was expected that the lac^+, pro_3^+, and ade_3^+ markers from a Hfr H $\phi,4^r pro^-_{1.2}$ donor would enter early like leu^+ but be unlinked to leu^+. It is obvious from the results presented in Fig. 1 and in Table 10 that this prediction was not borne out. The only justified conclusion from the experiments with the Hfr H $\phi,4^r pro^-_{1.2}$ mutants was that the aberrations associated with these $\phi,4^r pro^-_{1.2}$ mutations were not inversions or transpositions.

One possible explanation for the results obtained with the Hfr H $\phi,4^r pro^-_{1.2}$ mutants would be that an Hfr H strain with a linear chromosome due to a break at the $\phi,4^r pro^-_{1.2}$ locus would probably be inviable, since, in Hfr H, chromosome replication proceeds sequentially from the attached F (Nagata, 1963). In contrast, Nagata (1963) showed that there was no single fixed origin for the initiation of chromosome replication in an F^- strain. Since F^- and F^+ $\phi,4^r pro^-_{1.2}$ mutants appear to have a linear chromosome with a single break at the $\phi,4^r pro^-_{1.2}$ locus and are viable, it would be predicted that chromosome replication in these mutants begins at one end of the break. Preliminary experiments support this, and a more rigorous test of this prediction is now being made. It is thus possible that the different results obtained in crosses with F^+ and F^- $\phi,4^r pro^-_{1.2}$ mutants as compared with those obtained with Hfr H $\phi,4^r pro^-_{1.2}$ mutants are due to a difference in the mode of chromosome

replication in F^+ and F^- strains as opposed to that in Hfr strains.

If the Hfr H $\phi,4^r pro^-_{1,2}$ mutants do have a circular chromosome with no break at the $\phi,4^r pro^-_{1,2}$ locus, then it must be explained why these mutants are T_4-resistant. As implied above, the association of T_4 resistance with a linear chromosome and T_4 sensitivity with a circular chromosome may be fortuitous. If the open chromosome does interfere with the functioning of a neighboring gene necessary for T_4 sensitivity, it is then also possible that a larger deletion could encompass this neighboring gene and give rise to a $\phi,4^r pro^-_{1,2}$ mutation which could have either a linear or a circular chromosome.

There is another possible explanation for the results obtained with the Hfr H $\phi,4^r pro^-_{1,2}$ mutants. Wollman and Jacob (1958) stated that, to obtain a fully fertile Hfr recombinant in a cross with an F^- strain, it was essential to integrate both the proximal and the distal regions of the Hfr chromosome. Thus, a strain with a distally attached F but with no origin would behave as an ineffective donor. Therefore, if, in an Hfr H $\phi,4^r pro^-_{1,2}$ strain, the chromosome was sometimes broken at the $\phi,4^r pro^-_{1,2}$ locus, then the lac^+ pro_3^+ ade_3^+ ... *attached F* segment, which would lack an origin, would transfer the lac^+ marker to an F^- recipient early but at an undetectable frequency. The chromosome fragment containing the origin and the leu^+ marker, but with no distally attached F, also might fail to be transferred. Thus, even if an Hfr H chromosome with a break at the $\phi,4^r pro^-_{1,2}$ locus could replicate, cells containing such chromosomes would be poor donors of their genetic material.

In the cross with the Hfr H $\phi,4^r pro^-_{1,2}$ mutant (Fig. 1 and Table 10), the amount of recombination between the $\phi,4^r pro^-_{1,2}$ and lac^+ markers was excessive. This suggested that the Hfr chromosome could break after transfer, so that the *lac* pro_3 ade_3 segment could sometimes be integrated by recombinants independently of the *leu* to $\phi,4^r pro_{1,2}$ region. [Recall that with F^+ $\phi,4^r pro^-_{1,2}$ donors the $\phi,4^r pro^-_{1,2}$ marker was inherited with leu^+ and not lac^+ (Table 9).] If this explanation is correct, then it should be pointed out that it cannot be determined whether all or only some of the transferred partial chromosomes broke at the $\phi,4^r pro^-_{1,2}$ locus after transfer. Taylor and Adelberg (1961) showed, in crosses between Hfr donors and F^- phenocopy Hfr recipients, that markers on the proximal portion of the donor chromosome were inherited at a frequency of 70% among recombinants inheriting a marker on the distal end of the donor chromosome. Similar results were reported by Wollman and Jacob (1958) for Hfr $\times$ F^- crosses. Thus, when an entire linear chromosome (Hfr) pairs with an entire circular chromosome (F^-), there is a linked inheritance of markers on either side of the break in the linear chromosome. Thus, it might be possible to obtain the observed 77% association in the inheritance of the $\phi,4^r pro^-_{1,2}$ and lac^+ Hfr markers (Table 10B), even if all of the transferred partial chromosomes from the Hfr H $\phi,4^r pro^-_{1,2}$ donor had breaks at the $\phi,4^r pro^-_{1,2}$ locus. It should be pointed out that there is no apparent reason why chromosome breakage should occur as a result of chromosome transfer.

The above discussion indicates that the results obtained with two Hfr H $\phi,4^r pro^-_{1,2}$ mutants are explanable on the basis of known mechanisms of Hfr chromosome replication and transfer. Thus, no substantial change is required in the original theory that $\phi,4^r pro^-_{1,2}$ mutations cause single breaks in the bacterial chromosome. It is proposed that both Hfr H $\phi,4^r pro^-_{1,2}$ mutants have circular chromosomes and that their $\phi,4^r pro^-_{1,2}$ mutations are deletions. Thus, it would be impossible to obtain T_4-sensitive revertants from these Hfr H $\phi,4^r pro^-_{1,2}$ mutants and, in fact, no T_4 sensitive revertants have been found.

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EDUCATION:

Graduated Rural Retreat High School, 1961.
Associate in Science, Wytheville Community College 1973
BS in Biochemistry, Virginia Polytechnic Institute & State University, 1975.

EMPLOYMENT HISTORY:

US Army March 1962 - September 1965
Construction: Heavy Equipment Operator, 1966 – March 1970
Injury from Automobile Accident & Convalescence, March 1970 – March 1971
Student, March 1971 – June 1975

Sybron Corporation, Biochemical Division, Salem, VA, 1975 – 1983

- R&D Technician – Development of Bacterial Cultures for the Breakdown of Industrial Chemicals
- Field Service Technician
- Operations/Facility Manager

Microbe Masters, Inc (Texas Based), September 1983 – April 1985

- Technical Services – Biological Waste Treatment

Self Employed (Texas Based Consultant), April 1985 – October 1985

- Consultant – Biological Waste Treatment

Analytichem International, Texas Based, October 1985 – October 1986

- Sales Representative for Sample Preparation Materials before chromatographic analysis

Self Employed, Texas Based, October 1986– October 1987

- Consultant in field of Microbiology
- Commissioned sales representative for Worldwide Monitoring Inc., for Sample Preparation Materials

UCT, LLC., Basic Manufacturer of Sample Preparation Materials, October 1987 – To present

- UCT was previously named Worldwide Monitoring Inc. & United Chemical Technologies, Inc.,
- Texas Sales Representative, 1987 - 1990
- Vice President of Operations at Bristol, PA 1990 – 1993
- Vice President of Sales and Marketing at Bristol, PA 1993 – 1996
- Sales Representative (Virginia Based) – Mid West and Southeast Territories 1996 – 2001
- Operations Manager (At Lewistown & Bristol, PA Locations), 2001 – 2002
- Sales Manager (Virginia Based), 2002 – 2004
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